(51) International Patent Classification 6 : A61K 38/02, 38/04, 38/16, C07K 7/06, 7/08	A1	(11) International Publication Number: WO 98/11907
		(43) International Publication Date: 26 March 1998 (26.03.98)

(21) International Application Number: PCT/US97/16217

(22) International Filing Date: 15 September 1997 (15.09.97)

(30) Priority Data:
60/026.978   20 September 1996 (20.09.96)   US
08/928,958   12 September 1997 (12.09.97)   US

(71) Applicant: BRISTOL-MYERS SQUIBB COMPANY [US/US]; Lawrenceville-Princeton Road, Princeton, NJ 08543-4000 (US).

(72) Inventors: NADLER, Steven, G.; 500 W. Roy Street #206W, Seattle, WA 98119-3874 (US). CLEAVELAND, Jeffrey, S.; 8300 32nd Avenue N.W., Seattle, WA 98107-3921 (US). BLAKE, James; 201 Galer Street #243, Seattle, WA 98109-3192 (US). HAFFAR, Omar, K.; 101 Elliott Avenue West, Seattle, WA 98119 (US).

(74) Agents: ROBINS, Roberta, L.; Robins & Associates, Suite 200, 90 Middlefield Road, Menlo Park, CA 94025 (US) et al.

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PEPTIDE INHIBITORS OF NUCLEAR PROTEIN TRANSLOCATION HAVING NUCLEAR LOCALIZATION SEQUENCES AND METHODS OF USE THEREOF

(57) Abstract

Novel polypeptide inhibitors of cytoplasmic protein nuclear translocation are disclosed. The inhibitors are comprised of a signal sequence and at least two nuclear localization sequences. The polypeptides are useful as immunosuppression, antiviral and antitumor agents.

5 PEPTIDE INHIBITORS OF NUCLEAR PROTEIN TRANSLOCATION
HAVING NUCLEAR LOCALIZATION SEQUENCES
AND METHODS OF USE THEREOF

Technical Field

10   The present invention relates generally to
polypeptide inhibitors of gene expression. More
particularly, the invention relates to polypeptide
inhibitors of nuclear protein translocation which have
gene expression modulating activity, immunosuppressive

15 activity, antiviral activity, and antitumor activity.

Background of the Invention

Nuclear transport is essential to a number
of biological processes including gene expression and

20 cell division, as well as to viral replication,
tumorigenesis and tumor cell proliferation. The
mechanism of nuclear transport has only recently been
characterized in detail and has been shown to involve
a number of discrete steps. Proteins that are

25 destined to be transported into the nucleus contain
within their amino acid sequence a short stretch of
amino acids termed a nuclear localization sequence
("NLS"). These sequences are generally basic in
nature, however, there has been no consensus sequence

30 identified. Thus, there is a wide variety of these
sequences that appear to be specific for particular
proteins.

Within the cell, these NLSs may be either
masked or unmasked by accessory proteins or by

35 conformational changes within the NLS-containing
protein. An NLS may be masked because it is buried in
the core of the protein and not exposed on the surface

of the protein. Unmasking of NLSs, and nuclear
translocation of cytoplasmic proteins may be triggered
by phosphorylation, dephosphorylation, proteolytic
digestion, subunit association or dissociation of an
5   inhibitory subunit, or the like. Accordingly, the
masking and unmasking of NLSs provides a mechanism by
which the transport of these cytoplasmic proteins into
the nucleus may be regulated.

Nuclear translocation of transcription
10   factors requires the presence of an unmasked or
activated NLS in the nucleus-targeted protein. The
binding of certain ligands to cell surface receptors
activates the nuclear translocation of cytoplasmic
transcription factors. Once in the nucleus, these
15   transcription factors exert gene expression modulatory
activity.

NF-κB is a ubiquitous transcription factor
found in various levels and states of activation in
different cell types. NF-κB is composed of several
20   different subunits including p65, p50, c-rel, p52 and
p105. Recent studies suggest that distinct NF-κB
complexes contribute to the regulatory control of gene
transcription. The function and regulation of NF-κB
has been most well-characterized in lymphocytic cells.
25   In these cells, there is a wide variety of target
genes, e.g., immunoregulatory genes, that are
regulated by NF-κB including κ Ig light chains. Such
genes include those that encode the interleukin-2α
("IL-2α") receptor, interleukin-2 ("IL-2"),
30   interleukin-6 ("IL-6"), tumor necrosis factor-α
("TNF-α"), and the like.

In unstimulated cells, a major form of NF-κB
is a heterodimer of p50 and p65 (RelA) subunits.
Nonactive NF-κB is retained in the cytoplasm as an
35   inactive complex by inhibitory proteins such as IκBα,
β and γ. When cells are appropriately stimulated,
e.g., by a proinflammatory stimulus such as a

cytokine, the IκBs are degraded, thereby releasing free NF-κB dimers, which translocate to the nucleus and activate target genes, e.g., lymphokine genes and other immunoregulatory genes. This response is

5 transient and is terminated through delayed NF-κB-mediated IκBα induction.

Recently it has been demonstrated that glucocorticoids exert their immunosuppressive activity by inhibiting NF-κB nuclear translocation. Scheinman

10 et al. (1995) *Science* 270:283-286 and Auphan et al. (1995) *Science* 270:286-290 independently demonstrated that the inhibition is mediated by an increase in the induction by glucocorticoids of IκB inhibitory proteins. These investigators proposed that

15 inhibitors of NF-κB may be useful immunosuppressive and anti-inflammatory agents. Such an NF-κB nuclear translocation inhibitor, comprising an NLS from the p50 subunit of NF-κB attached to a membrane-permeable polypeptide motif, was described in Lin et al. (1995)

20 *J. Biol. Chem.* 270:14255-14258.

Nuclear translocation of proteins other than endogenous transcription factors and other cytoplasmic proteins also depends on the presence of an activated or unmasked NLS. For example, nuclear translocation

25 of the retroviral preintegration complex is a crucial step in human immunodeficiency virus type-1 ("HIV-1") replication in nondividing cells such as monocytes and growth-arrested T cells. Such translocation is dependent on the presence of an NLS in the N-terminal

30 portion of HIV matrix antigen ("MA") p17. Indeed, the HIV-1 enhancer contains tandem binding sites for NF-κB that can be essential for virus replication (Ross et al. (1991) *J. Virol.* 65:4350-4358; Parrott et al. (1991) *J. Virol* 65:1414-1419). Nuclear translocation

35 of the HIV-1 preintegration complex can be partially inhibited by an excess of the SV40 large T antigen NLS (Gulizia et al. (1994) *J. Virol.* 68:2021-2025).

Furthermore, Dubrovsky et al. (1995) *Molecular Med.*
2:217-230 reported that a series of compounds capable
of binding to and reacting with the HIV-1 MA p17 NLS
inhibit HIV-1 replication in human monocytes.

5   In addition, tumorigenesis and tumor cell
proliferation are regulated by the expression of
oncoproteins, many of which are cytoplasmic
transcription factors that are translocated into the
nucleus by virtue of the presence of an NLS. Miller
10   et al. (1996) *J. Cell Biochemistry* 60:560.

Accordingly, inhibitors of nuclear
translocation of cytoplasmic proteins would be useful
as gene expression modulating agents, immunoregulatory
agents, antiviral agents, antitumor agents, and the
15   like.

                    Summary of the Invention

The present invention provides for a
polypeptide that can be introduced into an intact cell
20   for the purpose of inhibiting the nuclear
translocation of a cytoplasmic protein. The
polypeptide contains at least two NLSs and an amino
acid sequence that can deliver the polypeptide through
the cytoplasmic membrane into the cell. The inventors
25   herein have found that such a polypeptide exhibits
surprisingly superior characteristics compared to a
polypeptide having only one NLS.

Accordingly, in one embodiment, the
invention is directed to a polypeptide comprising a
30   signal sequence peptide and at least two NLSs
covalently attached thereto.

In another embodiment, the invention is
directed to a method of introducing an exogenous
polypeptide comprising an NLS into an intact cell to
35   inhibit nuclear translocation of a cellular protein.
The method includes providing a polypeptide comprising
a signal sequence peptide and at least two NLSs as

described above, and contacting the cell with the polypeptide for a period of time effective to introduce the exogenous polypeptide into the cell.

5   In still another embodiment, the invention is directed to a method of suppressing an immune response of a subject comprising administering to the subject an immunosuppressive amount of a polypeptide comprising a signal sequence peptide and at least two NLSs.

10   In a further embodiment, the invention is directed to a method of treating or preventing a viral infection in an individual comprising administering to the individual an effective antiviral amount of a polypeptide inhibitor of nuclear translocation of a cellular protein, said inhibitor comprising a signal

15   sequence peptide and at least two NLSs.

In yet a further embodiment, the invention is directed to a method of transcriptionally modulating the expression of cellular genes comprising

20   contacting a target cell with an inhibitor of nuclear translocation of a cellular protein, wherein the inhibitor comprises a signal sequence peptide and at least two NLSs.

These and other embodiments of the subject

25   invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

FIG. 1A is a graphical representation of the

30   effect of PKKKRKVAAVALLPAVLLALLAPKKKRKVC (SEQ ID NO:1) (the "SV40MEM" polypeptide) on lipopolysaccharide ("LPS")-stimulated surface antigen expression in 70Z/3 murine leukemia pre-B cells (solid bar: κ Ig light chain; stippled bar: IL-2α receptor; striped bar:

35   CD45). FIG. 1B is a graphical representation of the effect of three inhibitory polypeptides on LPS-stimulated κ Ig light chain production in 70Z/3 murine

leukemia pre-B cells (diamonds: the SV40MEM
polypeptide; squares: KKKYKAAVALLPAVLLALLAKKKYKC (SEQ
ID NO:2) (the "HIV-1MEM" polypeptide); triangles:
AKRVKLAAVALLPAVLLALLAKRVKLC (SEQ ID NO:3) (the "C-
5   MYCMEM" polypeptide)). **FIG. 1C** is a graphical
representation of the effect of increasing LPS
concentrations on SV40MEM-inhibited κ Ig light chain
production in 70Z/3 murine leukemia pre-B cells.

        FIG. 2 is a graphical representation of the
10   effect of the SV40MEM polypeptide on LPS-stimulated
cytokine production in 70Z/3 murine leukemia pre-B
cells (squares: untreated; diamonds: the SV40MEM
polypeptide (10 μM); circles: the SV40MEM polypeptide
(5 μM); triangles: the SV40 NLS (10 μM)).

15   **FIG. 3** is a graphical representation of
dose-response relationships of three inhibitory
polypeptides on LPS-stimulated κ Ig light chain
production in 70Z/3 murine leukemia pre-B cells
(triangles: a peptide containing only the fibroblast
20   growth factor ("FGF") signal sequence AAVALLPAVLLALLAP
(SEQ ID NO:4) (the "MEM" peptide); squares: a
polypeptide having the FGF signal sequence and the NLS
of NF-κB p50 on the carboxy terminus of the signal
sequence, namely, AAVALLPAVLLALLAPVQRKRQKLMP (SEQ ID
25   NO:5) polypeptide (the "NF-κBMEM" polypeptide);
diamonds: the SV40MEM polypeptide comprised of L-amino
acids; circles: the SV40MEM polypeptide comprised of
D-amino acids.).

        FIG. 4A is a graphical representation of the
30   effect of inhibitory polypeptides on LPS-stimulated
TNF-α production. **FIG. 4B** is a graphical
representation of the effect of inhibitory
polypeptides on LPS-stimulated interleukin-8 ("IL-8")
production. In both **FIG. 4A** and **FIG. 4B** the following
35   symbols are used: squares - media control; crossed
squares - the MEM peptide (5 μM); diamonds - the NF-
κBMEM polypeptide (5 μM); circles - the SV40MEM

-6-

polypeptide comprised of L-amino acids (5 μM); and
triangles - the SV40MEM polypeptide comprised of D-
amino acids (5 μM).

5   **FIG. 5** is a graphical representation of the
effect of the SV40MEM polypeptide on LPS-induced CD40
expression in 70Z/3 murine leukemia pre-B cells.

FIG. 6A is a graphical representation of the
effect of the HIV-1MEM polypeptide on ^{3}H-
deoxyribothymidine uptake into peripheral blood
10   mononuclear cells ("PBMCs"). **FIG. 6B** is a graphical
representation of the effect of HIV-1MEM on viral p24
production in anti-CD3 stimulated PBMCs infected with
HIV-1 primary isolate M1. **FIG. 6C** is a photograph of
a gel depicting the effect of HIV-1MEM polypeptide on
15   the expression of proviral gag sequences in anti-CD3
activated PBMCs infected with HIV-1 primary isolate
M1.

FIG. 7 is a photograph of a gel depicting
the results of a polymerase chain reaction analysis of
20   the effect of HIV-1MEM polypeptide on the expression
of proviral gag sequences in H9 human lymphoma T-cells
or Jurkat human leukemia T-cells infected with HIV-1
primary isolate M1.

FIG. 8A is a photograph of gels depicting
25   the results of polymerase chain analyses of the effect
of HIV-1MEM and the NF-κBMEM polypeptide on the
expression of proviral gag sequences in Jurkat T-cells
infected with HIV$_{LAI}$. **FIG. 8B** is a photograph of gels
depicting the results of polymerase chain analyses of
30   the effect of HIV-1MEM and the NF-κBMEM polypeptide on
the expression of 2-long terminal repeat ("LTR")
circles in Jurkat T-cells infected with HIV$_{LAI}$.

FIG. 9 is a graphical representation of the
effect of SV40MEM prepared from D-amino acids on
35   proliferation of 70Z/3 murine leukemia pre-B cells.

FIG. 10 is a graphical representation of the effect of SV40MEM prepared from D-amino acids on proliferation of RAJI human B-cell leukemia cell line.

FIG. 11A is a graphical representation of the effect of an intravenous administration of the SV40MEM polypeptide on the *in vivo* response of mice to sheep red blood cells. FIG. 11B is a graphical representation of the effect of an oral administration of the SV40MEM polypeptide on the *in vivo* response of mice to sheep red blood cells.

FIG. 12 shows the effect of BMS 205820 and C-MYCMEM (BMS 214572) on the anti-hemocyanin (KLH) response in mice.

FIG. 13A, shows the effect of the BMS 205820 polypeptide on the production of TNF-α *in vivo*. FIG. 13B shows the effect of the BMS 205820 polypeptide on the production of IL-6 *in vivo*. FIG. 13C shows the effect of the BMS 205820 polypeptide on the production of IL-10 *in vivo*.

FIG. 14 depicts the effect of BMS 205820, C-MYCMEM (BMS 214572), an SV40 NLS polypeptide containing a single NLS, a c-myc NLS alone, without a translocation sequence (AKRVKL (SEQ ID NO:6)) and a control non-NLS polypeptide, 377G, on lipopolysaccharide (LPS) binding to CD14.

FIG. 15A is a time line depicting the administration scheme for the intraperitoneal injection of ovalbumin (OVA), nebulized OVA, BMS 205820 and C-MYCMEM. FIG. 15B shows the % eosinophils in lung following the treatment outlined in FIG. 15A.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of protein chemistry and biochemistry, molecular biology, microbiology and recombinant DNA technology, which are within the skill of the art.

Such techniques are explained fully in the literature.
See, e.g., Sambrook, Fritsch & Maniatis, *Molecular
Cloning: A Laboratory Manual,* Second Edition (1989);
DNA Cloning, Vols. I and II (D.N. Glover ed. 1985);

5   *Oligonucleotide Synthesis* (M.J. Gait ed. 1984);
Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins
eds. 1984); *Animal Cell Culture* (R.K. Freshney ed.
1986); *Immobilized Cells and Enzymes* (IRL press,
1986); Perbal, B., *A Practical Guide to Molecular*

10   *Cloning* (1984); the series, *Methods In Enzymology* (S.
Colowick and N. Kaplan eds., Academic Press, Inc.).

As used in this specification and the
appended claims, the singular forms "a," "an" and
"the" include plural references unless the content

15   clearly dictates otherwise.

A.   <u>Definitions</u>

In describing the present invention, the
following terms will be employed, and are intended to

20   be defined as indicated below.

As used herein, the term "signal sequence"
or "signal sequence peptide" is used to indicate a
peptide that is capable of directing the movement of
the polypeptide of which it is a part through a cell

25   membrane. In particular, the term is used to indicate
a peptide that directs the movement of a polypeptide
across the cytoplasmic membrane into the cell. The
term "signal sequence" is intended to encompass not
only the signal sequence of a particular polypeptide,

30   but also fragments or derivatives thereof that are
capable of delivering a polypeptide through a cell
membrane. A "signal sequence" may be composed of L-
or D-amino acids.

The terms "nuclear localization sequence"

35   and "NLS" are used interchangeably to indicate a
peptide that directs the transport of a protein with
which it is associated from the cytoplasm of a cell

across the nuclear envelope barrier. The term "NLS"
is intended to encompass not only the nuclear
localization sequence of a particular peptide, but
also derivatives thereof that are capable of directing
5   translocation of a cytoplasmic polypeptide across the
nuclear envelope barrier. NLSs are capable of
directing nuclear translocation of a polypeptide when
attached to the N-terminus, the C-terminus, or both
the N- and C- termini of the polypeptide. In
10   addition, a polypeptide having an NLS coupled by its
N- or C-terminus to amino acid side chains located
randomly along the amino acid sequence of the
polypeptide will be translocated. Adam et al. (1990)
J. Cell. Biol. 111:807-818. "Nuclear localization
15   sequences" may be composed of D- or L-amino acids.

By "interchangeably flanked at its amino-
and carboxy-termini by a first and a second NLS" is
intended to mean that the first or second NLS may be
located at either the amino- or carboxy-terminus of
20   the signal sequence polypeptide.

An "inhibitor of nuclear translocation" is a
polypeptide composed of a signal sequence peptide and
at least two NLSs which inhibits, e.g., either
decreases or halts, nuclear localization of a
25   cytoplasmic protein. Preferably, the polypeptide
comprises a signal sequence peptide interchangeably
flanked at its amino- and carboxy-termini by a first
and a second NLS. The NLSs at the N- and C-termini
may be the same or different. In one preferred
30   embodiment, the signal sequence peptide and the NLSs
are each composed of L-amino acids. In another
preferred embodiment, the signal sequence peptide and
the NLSs are each composed of D-amino acids.

A "derivative" of a polypeptide is intended
35   to include homologous polypeptides in which
conservative amino acid substitutions have been made,
as well as to include other amino acid substitutions

that result in a polypeptide that retains its
function, e.g., as a signal sequence peptide, an NLS,
or an inhibitor of nuclear localization. A
"derivative" of a peptide may be a peptide mimetic.

5   "Peptide mimetics" are structures which
serve as substitutes for peptides in interactions with
acceptor molecules (see Morgan et al. (1989) *Ann.
Reports Med. Chem.* 24:243-252 for a review of peptide
mimetics). Peptide mimetics, as used herein, include

10   synthetic structures which may or may not contain
amino acids and/or peptide bonds, but retain the
structural and functional features of a peptide
ligand. The term, "peptide mimetics" also includes
peptoids and oligopeptoids, which are peptides or

15   oligomers of N-substituted amino acids (Simon et al.
(1972) *Proc. Natl. Acad. Sci. USA* 89:9367-9371).
Further included as peptide mimetics are peptide
libraries, which are collections of peptides designed
to be of a given amino acid length and representing

20   all conceivable sequences of amino acids corresponding
thereto. Methods for the production of peptide
mimetics are described more fully below.

          Two polypeptide sequences are "substantially
homologous" when at least about 85% (preferably at

25   least about 85% to 90%, and most preferably at least
about 95%) of the nucleotides or amino acids match
over a defined length of the molecule. As used
herein, substantially homologous also refers to
sequences showing identity to the specified

30   polypeptide sequence.

          The terms "polypeptide", "peptide" and
"protein" are used interchangeably and refer to any
polymer of amino acids (dipeptide or greater) linked
through peptide bonds. Thus, the terms "polypeptide",

35   "peptide" and "protein" include oligopeptides, protein
fragments, analogues, muteins, fusion proteins and the
like.

-11-

The following single-letter amino acid abbreviations are used throughout the text:

Alanine	A	Arginine	R
Asparagine	N	Aspartic acid	D
Cysteine	C	Glutamine	Q
Glutamic acid	E	Glycine	G
Histidine	H	Isoleucine	I
Leucine	L	Lysine	K
Methionine	M	Phenylalanine	F
Proline	P	Serine	S
Threonine	T	Tryptophan	W
Tyrosine	Y	Valine	V

By an "isolated polypeptide" is meant a polypeptide which is devoid of, in whole or part, tissue or cellular components with which the protein is normally associated in nature. Thus, a polypeptide contained in a tissue extract would constitute an "isolated" polypeptide, as would a polypeptide synthetically or recombinantly produced.

By "mammalian subject" is meant any member of the class Mammalia, including, without limitation, humans and non-human primates, such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; and laboratory animals including rodents such as mice, rats and guinea pigs. The term does not denote a particular age. Thus, adult, newborn and fetal mammals are intended to be covered.

The term "treatment" as used herein refers to either (i) the prevention of infection or reinfection (prophylaxis), or (ii) the reduction or elimination of symptoms of the disease of interest (therapy).

B. General Methods

Central to the present invention is the
discovery of polypeptide molecules that inhibit
nuclear localization of cytoplasmic proteins. These
5   molecules comprise a signal sequence peptide and at
least two NLSs. The polypeptide inhibitors, and
derivatives thereof, provide useful tools for
introducing an exogenous polypeptide comprising an NLS
into an intact cell to inhibit nuclear translocation
10   of a cellular protein, for studying the role of
nuclear translocation in the regulation of cellular
processes.

In addition, since the nuclear translocation
of certain cellular peptides is required for the host
15   organism to mount an immune response, the polypeptide
inhibitors are useful as immunosuppression agents.
Immune responses are typically manifested by the
expression of antibodies, the production of a number
of cytokines, and/or the expression of cell surface
20   receptors. Thus, inhibition of immune responses by
the inhibitory peptides can take the form of:
inhibition of antibody production, including the
production of antibody component peptides such as a κ
light chain polypeptide; inhibition of cytokine
25   production, including such cytokines as interleukin-1,
interleukin-2, interleukin-4, interleukin-6,
interleukin-10, tumor necrosis factor, or granulocyte-
macrophage colony-stimulating factor; and/or the
inhibition of the expression of cell-surface receptors
30   such as an interleukin-2 receptor, gp39, CD40, CD45,
CD80, CD86, ICAM, ELAM, major histocompatibility
complex ("MHC") class II, or VCAM. Clark et al.
(1994) *Nature* 367:425.

By virtue of their immunosuppressive
35   activities, the polypeptide inhibitors of the present
invention are useful in the treatment of a wide
variety of immune disorders, including but not limited

-13-

to, the treatment of autoimmune diseases such as
rheumatoid arthritis, multiple sclerosis, juvenile-
onset diabetes, systemic lupus erythematosus (SLE),
autoimmune uveoretinitis, autoimmune vasculitis,
bullous pemphigus, myasthenia gravis, autoimmune
thyroiditis or Hashimoto's disease, Sjogren's
syndrome, granulomatous orchitis, autoimmune
oophoritis, Crohn's disease, sarcoidosis, rheumatic
carditis, ankylosing spondylitis, Grave's disease, and
autoimmune thrombocytopenic purpura (see e.g., Paul,
W.E. (1993) *Fundamental Immunology*, Third Edition,
Raven Press, New York, Chapter 30, pp. 1033-1097; and
Cohen et al. (1994) *Autoimmune Disease Models, A
Guidebook*, Academic Press, 1994).

Similarly, the polypeptide inhibitors of the
present invention are useful for treating physical
symptoms manifested by responses to allergens which
can initiate a state of hypersensitivity, or which can
provoke a hypersensitivity reaction in a subject
already sensitized with the allergen. Such physical
symptoms include asthma, joint swelling, urticaria,
and the like. Additionally, due to their
immunosuppressive properties, the polypeptide
inhibitors of the present invention are useful in the
treatment of sepsis and in the prevention of septic
shock, a potentially lethal condition caused by the
uncontrolled production of certain cytokines due to
the presence of endotoxins, such as lipopolysaccharide
(LPS), from extracellular bacteria.

Furthermore, since many viruses, e.g.,
herpes virus, cytomegalovirus, retroviruses, and the
like, make use of the host cell's nuclear
translocation machinery, the inhibitory polypeptides
are useful as antiviral agents. In addition, since
tumorigenesis and tumor cell proliferation appear to
be mediated by the expression of oncogenes to make
oncoproteins, many of which are transcription factors

-14-

that are translocated into the nucleus, Miller et al.
(1996), *supra*, the polypeptide inhibitors, or
derivatives thereof, can be used to suppress tumor
growth.

5   The claimed inhibitors include sequences of
amino acids that comprise signal sequences from such
polypeptides as the antennapedia homeodomain, FGF, HIV
Tat, or Hsc70, and derivatives or mimetics thereof
capable of delivering the inhibitor through the
10   cytoplasmic membrane into the cell. Preferred signal
sequences include RQIKIWFQNRRMKWKK (SEQ ID NO:7),
AAVALLPAVLLALLA (SEQ ID NO:8), AAVALLPAVLLALLAP (SEQ
ID NO:4), CFITKALGISYGRKKRRQRRRPPQGSQTH (SEQ ID NO:9),
and the like, or derivatives or mimetics thereof
15   capable of delivering the inhibitor through the
cytoplasmic membrane into the cell.

          Candidate signal sequences can be tested for
their ability to direct the translocation of proteins
across cell membranes, for example, by monitoring the
20   localization of exogenous detectably labeled proteins
into the cell cytoplasm. Lin et al. (1995), *supra*,
describe the use of radiolabeled proteins. *In vitro*
nuclear peptide import can be measured using NLS
peptides coupled to a fluorescent protein by methods
25   described in Adam et al. (1990), *supra*.

          The polypeptide inhibitor further comprises
at least two NLSs. The NLSs can be covalently bonded
to the N-terminus, to the C-terminus, to both the N-
and C- termini of the signal sequence polypeptide, to
30   amino acid side chains located along the amino acid
sequence of the signal sequence polypeptide, or any
combination thereof. Preferably, the signal sequence
polypeptide is interchangeably flanked at its amino-
and carboxy-termini by a first and a second NLS. The
35   first and second NLSs may be the same or different. A
discussion of NLSs and a list of NLSs can be found in

Boulikas (1993) *Crit. Rev. Eukaryotic Gene Expression*
3:193-227, and references cited therein.

Approaches for identifying NLSs include: (1)
gene fusion experiments between a candidate NLS-coding
5   DNA segment and the gene coding for a cytoplasmic
protein (*see, e.g.*, Silver et al. (1984) *Proc. Natl.*
Acad. Sci. U.S.A. *81*:5951; Moreland et al. (1987) *Mol.*
Cell. Biol. *7*:4048; and Picard et al. (1987) *EMBO J.*
6:3333); (2) nuclear import of nonnuclear proteins
10   conjugated to synthetic NLS peptides (*see, e.g.*,
Goldfarb et al. (1986) *Nature* *322*:641; Markland et al.
(1987) *Mol. Cell. Biol.* *7*:4255; and Chelsky et al.
(1989) *Mol. Cell. Biol.* *9*:2487); and (3) site-directed
mutagenesis of a specific segment of a nuclear
15   protein, resulting in its cytoplasmic retention (*see,*
e.g., Greenspan et al. (1988) *J. Virol.* *62*:3020; van
Etten et al. (1989) *Cell* *58*:669; and Boulukos et al.
(1989) *Mol. Cell. Biol.*.*9*:5718).

Preferred NLSs include PKKKRKV (SEQ ID
20   NO:10) and KKKRKVC (SEQ ID NO:11) from the SV40 large
T antigen (*see,* Kalderon et al. (1984) *Cell* *39*:499),
GKKRSKA (SEQ ID NO:12) from yeast histone H2B (*see,*
Moreland et al. (1987) *Mol. Cell. Biol.* *7*:4048), KRPRP
(SEQ ID NO:13) from adenovirus E1A (*see,* Lyons et al.
25   (1987) *Mol. Cell. Biol.* *7*:2451), GNKAKRQRST (SEQ ID
NO:14) from the *v-rel* oncogene of the avian
reticuloendotheliosis retrovirus strain T (*see,*
Gilmore et al. (1988) *J. Virol.* *62*:703), GGAAKRVKLD
(SEQ ID NO:15) from the human c-myc oncoprotein (*see,*
30   Chelsky et al. (1989) *Mol. Cell. Biol.* *9*:2487),
SALIKKKKKMAP (SEQ ID NO:16) from the murine *c-abl (IV)*
gene product (*see,* Van Etten et al. (1989) *Cell*
58:669), RKLKKLGN (SEQ ID NO:17) from the human or rat
androgen receptor (*see,* Guiochon-Mantel et al. (1989)
35   *Cell* *57*:1147), PQPKKKP (SEQ ID NO:18) from protein p53
(*see,* Dang et al. (1989) *J. Biol. Chem.* *264*:18019)),
ASKSRKRKL (SEQ ID NO:19) from viral *Jun*, a

transcription factor of the AP-1 complex (*see*, Chida
et al. (1992) *Proc. Natl. Acad. Sci. USA* <u>89</u>:4290),
KKKYK (SEQ ID NO:20) and KKKYKC (SEQ ID NO:21), both
of which are from the human immunodeficiency virus

5   matrix protein (*see*, Bukrinsky et al. (1993) *Nature*
<u>365</u>:666), KSKKK (SEQ ID NO:22) from the human
immunodeficiency virus matrix 2 protein (*see*,
Bukrinsky et al. (1993), *supra*), AKRVKL (SEQ ID NO:6)
and KRVKLC (SEQ ID NO:23) both of which are from the

10   human *c-myc* oncoprotein (*see*, Chelsky et al. (1989),
supra), and derivatives and mimetics thereof that are
effective as an NLS.

         The polypeptide inhibitors of the present
invention may be synthesized by conventional

15   techniques known in the art, for example, by chemical
synthesis such as solid phase peptide synthesis. Such
methods are known to those skilled in the art. In
general, these methods employ either solid or solution
phase synthesis methods, well known in the art. *See*,

20   *e.g.*, J. M. Stewart and J. D. Young, *Solid Phase
Peptide Synthesis*, 2nd Ed., Pierce Chemical Co.,
Rockford, IL (1984) and G. Barany and R. B.
Merrifield, *The Peptides: Analysis, Synthesis,
Biology*, editors E. Gross and J. Meienhofer, Vol. 2,

25   Academic Press, New York, (1980), pp. 3-254, for solid
phase peptide synthesis techniques; and M. Bodansky,
Principles of Peptide Synthesis, Springer-Verlag,
Berlin (1984) and E. Gross and J. Meienhofer, Eds.,
The Peptides: Analysis, Synthesis, Biology, *supra*,

30   Vol. 1, for classical solution synthesis.

         In general, these methods comprise the
sequential addition of one or more amino acids or
suitably protected amino acids to a growing peptide
chain. Normally, either the amino or carboxyl group

35   of the first amino acid is protected by a suitable
protecting group. The protected or derivatized amino
acid can then be either attached to an inert solid

-17-

support or utilized in solution by adding the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected, under conditions suitable for forming the amide linkage.

5   The protecting group is then removed from this newly added amino acid residue and the next amino acid (suitably protected) is then added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining protecting groups
10   and any solid support are removed either sequentially or concurrently to afford the final polypeptide. By simple modification of this general procedure, it is possible to add more than one amino acid at a time to a growing chain, for example, by coupling (under
15   condition that do not racemize chiral centers) a protected tripeptide with a properly protected dipeptide to form, after deprotection, a pentapeptide.

Typical protecting groups include *t*-butyloxycarbonyl (Boc), 9-fluorenylmethoxycarbonyl
20   (Fmoc), benxyloxycarbonyl (Cbz), *p*-toluenesulfonyl (Tos); 2,4-dinitrophenyl, benzyl (Bzl), biphenyl-isopropyloxycarboxycarbonyl, cyclohexyl, isopropyl, acetyl, *o*-nitrophenylsulfonyl, and the like. Of these, Boc and Fmoc are preferred.

25   Typical solid supports are generally cross-linked polymeric materials. These include divinylbenzene cross-linked styrene-based polymers, for example, divinylbenzene-hydroxymethylstyrene copolymers, divinylbenzene-chloromethylstyrene
30   copolymers, and divinylbenzene-benzhydrylaminopolystyrene copolymers. The Divinylbenzene-benzhydrylaminopolystyrene copolymers, as illustrated herein using *p*-methyl-benzhydrylamine resin, offers the advantage of directly introducing a
35   terminal amide functional group into the peptide chain, which function is retained by the chain when the chain is cleaved from the support.

In one preferred method, the polypeptides are prepared by conventional solid phase chemical synthesis on, for example, an Applied Biosystems, Inc. (ABI) 430A peptide synthesizer using a resin that

5 permits the synthesis of the amide peptide form and using t-Boc amino acid derivatives (Peninsula Laboratories, Inc.) with standard solvents and reagents. Polypeptides containing either L- or D-amino acids may be synthesized in this manner. Polypeptide

10 composition is confirmed by quantitative amino acid analysis and the specific sequence of each peptide may be determined by sequence analysis.

Alternatively, the polypeptides can be produced by recombinant DNA techniques by synthesizing

15 DNA encoding the desired polypeptide, along with an ATG initiation codon. Once coding sequences for the desired polypeptides have been synthesized or isolated, they can be cloned into any suitable vector for expression. Numerous cloning vectors are known to

20 those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage λ (*E. coli*), pBR322 (*E. coli*), pACYC177

25 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*),

30 YIp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine papilloma virus (mammalian cells). *See, generally, DNA Cloning*: Vols. I & II, *supra*; Sambrook et al., *supra*; B. Perbal, *supra*. Insect cell expression systems, such as baculovirus systems, can also be used

35 and are known to those of skill in the art and described in, *e.g.*, Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555*

●   ●

(1987). Materials and methods for baculovirus/insect
cell expression systems are commercially available in
kit form from, *inter alia*, Invitrogen, San Diego CA
("MaxBac" kit).

5   The gene can be placed under the control of
a promoter, ribosome binding site (for bacterial
expression) and, optionally, an operator (collectively
referred to herein as "control" elements), so that the
DNA sequence encoding the desired polypeptide is
10   transcribed into RNA in the host cell transformed by a
vector containing this expression construction. The
coding sequence may or may not contain a signal
peptide or leader sequence. Heterologous leader
sequences can be added to the coding sequence which
15   cause the secretion of the expressed polypeptide from
the host organism. Leader sequences can be removed by
the host in post-translational processing. *See, e.g.,*
U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.
            Other regulatory sequences may also be
20   desirable which allow for regulation of expression of
the protein sequences relative to the growth of the
host cell. Such regulatory sequences are known to
those of skill in the art, and examples include those
which cause the expression of a gene to be turned on
25   or off in response to a chemical or physical stimulus,
including the presence of a regulatory compound.
Other types of regulatory elements may also be present
in the vector, for example, enhancer sequences.
            The control sequences and other regulatory
30   sequences may be ligated to the coding sequence prior
to insertion into a vector, such as the cloning
vectors described above. Alternatively, the coding
sequence can be cloned directly into an expression
vector which already contains the control sequences
35   and an appropriate restriction site.
            In some cases it may be necessary to modify
the coding sequence so that it may be attached to the

control sequences with the appropriate orientation;
i.e., to maintain the proper reading frame. It may
also be desirable to produce mutants or analogs of the
polypeptide of interest. Mutants or analogs may be
5   prepared by the deletion of a portion of the sequence
encoding the protein, by insertion of a sequence,
and/or by substitution of one or more nucleotides
within the sequence. Techniques for modifying
nucleotide sequences, such as site-directed
10 mutagenesis, are well known to those skilled in the
art. *See, e.g.*, Sambrook et al., *supra; DNA Cloning*,
Vols. I and II, *supra; Nucleic Acid Hybridization*,
supra.

The expression vector is then used to
15 transform an appropriate host cell. A number of
mammalian cell lines are known in the art and include
immortalized cell lines available from the American
Type Culture Collection (ATCC), such as, but not
limited to, Chinese hamster ovary (CHO) cells, HeLa
20 cells, baby hamster kidney (BHK) cells, monkey kidney
cells (COS), human hepatocellular carcinoma cells
(*e.g.*, Hep G2), Madin-Darby bovine kidney ("MDBK")
cells, as well as others. Similarly, bacterial hosts
such as *E. coli, Bacillus subtilis*, and *Streptococcus*
25 *spp.*, will find use with the present expression
constructs. Yeast hosts useful in the present
invention include *inter alia, Saccharomyces
cerevisiae, Candida albicans, Candida maltosa,
Hansenula polymorpha, Kluyveromyces fragilis,
30 Kluyveromyces lactis, Pichia guillerimondii, Pichia
pastoris, Schizosaccharomyces pombe* and *Yarrowia
lipolytica.* Insect cells for use with baculovirus
expression vectors include, *inter alia, Aedes aegypti,
Autographa californica, Bombyx mori, Drosophila
35 melanogaster, Spodoptera frugiperda,* and *Trichoplusia
ni.* The proteins may also be expressed in
Trypanosomes.

Depending on the expression system and host
selected, the proteins of the present invention are
produced by growing host cells transformed by an
expression vector described above under conditions

5   whereby the protein of interest is expressed. The
protein is then isolated from the host cells and puri-
fied. If the expression system secretes the protein
into growth media, the protein can be purified
directly from the media. If the protein is not

10   secreted, it is isolated from cell lysates. The
selection of the appropriate growth conditions and
recovery methods are within the skill of the art.
Once purified, the amino acid sequences of the
proteins can be determined, i.e., by repetitive cycles

15   of Edman degradation, followed by amino acid analysis
by HPLC. Other methods of amino acid sequencing are
also known in the art.

       As explained above, peptide mimetics which
structurally and functionally mimic the peptide

20   inhibitors described above will also find use herein
and may be generated using the following strategies
and procedures. Generally, mimetics are designed
based on information obtained by systematic
replacement of L-amino acids by D-amino acids or, in

25   the case of a polypeptide inhibitor that is made of D-
amino acids, the systematic replacement of D-amino
acids by L-amino acids, replacement of side chain
moieties by a methyl group or pseudoisosteric groups
with different electronic properties (see Hruby et al.

30   (1990) Biochem. J. 268:249-262), and by systematic
replacement of peptide bonds in the above described
polypeptide inhibitors with amide bond replacements.
For example, analogues containing amide bond
surrogates may be used to investigate aspects of

35   peptide structure and function, such as rotational
freedom in the backbone, intra- and intermolecular
hydrogen-bond patterns, modifications of local and

-22-

total polarity and hydrophobicity, and oral
bioavailability.

Local conformational constraints can also be
introduced to determine conformational requirements

5   for activity of a candidate polypeptide mimetic
inhibitor of nuclear translocation. For example, β,β-
disubstituted amino acids may be used to examine the
effects of conformational constraints on peptide
activity (see, e.g., Manning et al. (1982) *J. Med.*

10   *Chem.* 25:408-414; Mosberg et al. (1983) *Proc. Natl.*
Acad. Sci. USA 106:506-512; Pelton et al. (1985) *Proc.*
Natl. Acad. Sci. USA 82:236-239).

The mimetics can include isosteric amide
bonds such as $\Psi[CH_2S]$, $\Psi[CH_2NH]$, $\Psi[CSNH_2]$, $\Psi[NHCO]$,

15   $\Psi[COCH_2]$ and $\Psi[(E)$ or (Z) $CH=CH]$ (see, for review,
Spatola (1983) in "Chemistry and Biochemistry of Amino
Acids, Peptides and Proteins," Volume VII, (Weinstein,
ed.), Marcel Dekker, New York, 267-357). Structures
which mimic the tetrahedral transition state

20   associated with hydrolysis of a substrate bond can
also be present and include hydroxymethylene,
fluoroketone moieties and phosphoramidate transition
state mimics (Bühlmayer et al. (1988) *J. Med. Chem.*
31:1839; Sham et al. (1988) *FEBS Lett.* 220:299;

25   Matthews (1988) *Acc. Chem. Res.* 21:333). Cyclic amino
acid analogues may be used to constrain amino acid
residues to particular conformational states, e.g.,
$\alpha\alpha'$- and $\beta\beta'$-substituted cyclic amino acids such as 1-
aminocyclopentanecarboxylic acid (cycloleucine) and

30   β,β-cyclopentamethylene-β-mercaptopropionic acid (see
Hruby et al. (1990), *supra*).

The mimetics can also include mimics of
inhibitor peptide secondary structure -- structures
which can model the 3-dimensional orientation of amino

35   acid residues into the known secondary conformations
of proteins -- including β-turn mimetics, such as
phenoxathin ring system, and β-sheet mimics, such as

-23-

epindolidione structures. Design, synthesis and
conformational analysis of an α-helix inducing
template has been described (Kemp et al.(1988)
Tetrahedron Lett. 29:4931; Kemp et al. (1988)
5   *Tetrahedron Lett.* 29:4935). Similarly, peptoids will
find use herein. Peptoids are oligomers of N-
substituted amino acids (Simon et al. (1972), *supra*),
and can be used as motifs for the generation of
chemically diverse libraries of novel molecules, which
10   can then be tested for nuclear translocation
inhibitory activity. The monomers may incorporate t-
butyl-based side-chain and 9-fluorenylmethoxy-carbonyl
α-amine protection. Oligomerization of the peptoid
monomers may be performed by, for example, *in situ*
15   activation by either benzotriazol-1-
yloxytris(pyrrolidino)phosphonium hexafluorphosphate
or bromotris(pyrrolidino)phosphonium
hexafluorophosphate. Other steps are identical to
conventional peptide synthesis using α-(9-
20   fluorenylmethoxycarbonyl)amino acids. Oligopeptoids
may be identified which have activities comparable to
the corresponding inhibitory polypeptides and, thus,
are useful as inhibitors of nuclear translocation (*see*
Simon et al. (1992), *supra*).
25   Peptide ligands that exhibit nuclear
translocation inhibitory activity can be developed by
using a biological expression system (*see* Christian et
al. (1992) *J. Mol. Biol.* 227:711-8; Devlin et al.
(1990) *Science* 249:404-406; Cwirla et al. (1990) *Proc.*
30   *Natl. Acad. Sci. USA* 87:6378-6382). The use of such
systems allows the production of large libraries of
random peptide sequences and the screening of these
libraries for peptide sequences that have desired
biochemical activity. The libraries may be produced
35   by cloning synthetic DNA that encodes random peptide
sequences into *Escherichia coli* expression vectors.
In the filamentous phage system, foreign peptide

sequences can be expressed on the surface of the
infectious phage (*see* Smith (1985) *Science* 228:1315-
1317; Parmley et al. (1988) *Gene* 73:305-318).

5   For example, a library may be made by
ligating into an appropriate phage, a synthetic DNA
fragment containing a degenerate coding sequence
$(NNK)_n$, where N stands for an equal mixture of the
deoxynucleotides G, A, T, and C, K stands for an
equimolar mixture of G and T, and n stands for the
10   number of amino acid residues desired in the product
peptide. Phage are screened for expression of
inhibitory activity. Those that express inhibitory
activity can be cloned and propagated, their DNAs
sequenced to determine the amino acid sequences of
15   their expressed polypeptide which can be assessed for
their ability to inhibit nuclear translocation.

          Large libraries of polypeptide inhibitors
can also be constructed by concurrent synthesis of
overlapping peptides as described in U.S. Patent No.
20   4,708,871 to Geysen. The solid support is generally a
polyethylene or polypropylene rod onto which is graft
polymerized a vinyl monomer containing at least one
functional group to produce polymeric chains on the
carrier. The functional groups are reacted to provide
25   primary or secondary amine groups which are
sequentially reacted with amino acid residues in the
appropriate order to build the desired synthetic
peptide using conventional methods of solid phase
peptide chemistry.

30   Once synthesized or otherwise produced, the
inhibitory activity of a candidate polypeptide or
polypeptide mimetic can be tested by assessing the
ability of the candidate to inhibit the
lipopolysaccharide-induced nuclear translocation of
35   NF-κB by, for example, using murine endothelial cells
by the method described in Lin et al. (1995), *supra*.

Inhibitory polypeptides can be used in pharmaceutical compositions to treat autoimmune diseases and viral infections, or to suppress transplant rejection. In addition, the polypeptides

5 can be administered to cancerous tissues in order to suppress tumor growth. The inhibitory polypeptides of the present invention can be formulated into therapeutic compositions in a variety of dosage forms such as, but not limited to, liquid solutions or

10 suspensions, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends upon the mode of administration and the particular cancer type targeted. The compositions

15 also preferably include pharmaceutically acceptable vehicles, carriers or adjuvants, well known in the art, such as human serum albumin, ion exchangers, alumina, lecithin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate,

20 and salts or electrolytes such as protamine sulfate. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. Actual methods of preparing such compositions are known, or will be apparent, to

25 those skilled in the art. *See, e.g., Remington's Pharmaceutical Sciences,* Mack Publishing Company, Easton, Pennsylvania, 18th edition, 1990.

The above compositions can be administered using conventional modes of delivery including, but

30 not limited to, intravenous, intraperitoneal, oral, intralymphatic, or subcutaneous administration. Local administration to a tumor in question, or to a site of inflammation, e.g., direct injection into an arthritic joint, will also find use with the present invention.

35 Therapeutically effective doses will be easily determined by one of skill in the art and will depend on the severity and course of the disease, the

patient's health and response to treatment, and the
judgment of the treating physician.

C.   Experimental

5   Below are examples of specific embodiments
for carrying out the present invention. The examples
are offered for illustrative purposes only, and are
not intended to limit the scope of the present
invention in any way.

10   Efforts have been made to ensure accuracy
with respect to numbers used (e.g., amounts,
temperatures, etc.), but some experimental error and
deviation should, of course, be allowed for.

15   Experimental Methods

Peptide Preparation: In general, polypeptides can be
synthesized using a stepwise solid-phase synthesis
method and purified on a C_{18} reverse phase high
20   performance liquid chromatography on a reverse-phase
column eluted with a linear gradient of 10%-60%
acetonitrile, 0.1% trifluoroacetic acid as described
in Merrifield (1963) J. Am. Chem. Soc. 85:2149-2154
and Lin et al. (1988) Biochemistry 27:5640-5645.
25   The peptides described herein were
synthesized on a Gilson AMS-422 multiple peptide
synthesizer using Fmoc amino acids. Gausepohl et al.
(1992) Peptide Res. 5:315-320. Peptides were cleaved
from the resin and deprotected by a 2-hr reaction with
30   trifluoroacetic acid/water/thioanisole/ethanedithiol
(100:5:5:2.5). the peptides were precipitated from
ether, redissolved in formic acid, diluted with water,
and lyophilized. Purification was achieved by
reverse-phase high performance liquid chromatography
35   using a gradient of acetonitrile in 0.1%
trifluoroacetic acid. All synthetic peptides were

characterized by mass spectroscopy on a Bio-Ion 20
instrument and gave the expected molecular weight.

Murine 70Z/3 pre-B leukemia cells (ATCC T1D-
158), H9 human lymphoma T-cells (ATCC CRL-8543),
Jurkat human leukemia T-cells (ATCC T1B-152), and THP-
1 human monocytic cells (ATCC T1B-202) were obtained
from the American Type Culture Collection.

Expression of κ Ig, CD45, CD40, and the
interleukin-2 receptor was assayed using standard
enzyme-linked immunosorbent assays ("ELISAs") (see,
Coligan et al. (eds.) (1991) Current Protocols in
Immunology, Wiley & Sons, p. 2.10 or by FACS staining
to measure cell-surface markers as described in Raff
(1970) Immunology 19:637). Antibodies form cell
surface markers were obtained from Pharmingen Co.

Amounts of TNF-α, IL-6, IL-8 and IL-10 were
determined using the ELISA kits obtained from Genzyme
following the manufacturer's instructions.

HIV-1 M1 p 24 levels were assayed using the
procedures described in Smithgall et al (1995) AIDS
Res. and Human Retrovirus 11:885. Proviral DNA
content (gag) was measured using polymerase chain
reaction analysis as described in Smithgall et al.
(1995), supra.

2-LTR circles, a strictly nuclear form of
HIV-1 DNA the expression of which indicates nuclear
localization of the viral genome, was measured by as
described in Bukrinsky et al (1991) Science 254:423.

Example 1
Inhibition by the SV40MEM Polypeptide of
κ Ig Light Chain Expression

This experiment was conducted to show that
the polypeptide PKKKRKVAAVALLPAVLLALLAPKKKRKVC (SEQ ID
NO:1) (the "SV40MEM" polypeptide), which comprises the
hydrophobic region of the signal peptide from
fibroblast growth factor and the SV40 large T antigen

NLS, has immunosuppressive activity. The effect of
the SV40MEM polypeptide was compared with two
additional polypeptides, KKKYKAAVALLPAVLLALLAKKKYKC
(SEQ ID NO:2) (the "HIV-1MEM" polypeptide), which is
5   composed of the membrane translocation domain of FGF
flanked by the NLS from the HIV-1 matrix protein and
AKRVKLAAVALLPAVLLALLAKRVKLC (SEQ ID NO:3) (the "C-
MYCMEM" polypeptide), which contains the NLS from the
human c-myc oncoprotein (Chelsky et al. (1989), *supra*,
10   and Dang et al. (1988), *supra*) using murine 70Z/3 pre-
B cells.

Murine 70Z/3 cells (ATCC T1D-158) are a
model cell line for analyzing the effects of compounds
on B-cell differentiation. In response to *S. typhosa*
15   LPS (Difco) or γ-interferon, these cells differentiate
from surface κ immunoglobulin negative to κ Ig
positive.

As seen in **FIG. 1A**, the SV40MEM polypeptide
caused an approximately 75-80% inhibition of κ light
20   chain expression in response to *S. typhosa* LPS (Difco)
after a 1-hr pretreatment with the SV40MEM
polypeptide. There was no effect of SV40MEM on the
expression of CD45 or the interleukin-2 receptor. The
maximum inhibitory effect occurred with an
25   approximately 3-hr SV40MEM pretreatment. This
inhibitory effect of the SV40MEM polypeptide can be
overcome by increasing the concentration of LPS.

FIG. 1B depicts dose-response data for the
effect of the D-amino acid form of the SV40MEM
30   polypeptide compared with L-amino acid forms of the
HIV-1MEM polypeptide and AKRVKLAAVALLPAVLLALLAKRVKLC
(SEQ ID NO:3). These data show that the three
polypeptides are approximately equally efficacious but
that the latter two polypeptides are not as potent at
35   inhibiting κ Ig light chain production as is SV40MEM.
FIG. 1C shows that increasing the concentration of LPS
can overcome the inhibitory effect of SV40MEM.

<u>Example 2</u>

<u>Inhibition by the SV40MEM Polypeptide of</u>

<u>Cytokine Production</u>

THP-1 control cells were harvested, washed
and set up in RPMI 1640, 10% fetal calf serum, at a
concentration of 2.56×10^6 cells/ml. Phosphate
buffered saline ("PBS") or one of the two respective
polypeptides SV40MEM or SV40 (a peptide having only
the SV40 large T antigen NLS sequence) were added to
the cells at a concentration of 10 μM or 20 μM. The
cells were then plated into a 96-well plate with 0.1
ml/well. The plate was then incubated for 2 hr at
37°C in a 5% CO_2 atmosphere. Following the initial
incubation, media or a titration of LPS was added to
each of the wells in a volume of 0.1 ml. Thus, the
final concentrations of polypeptides were 5 μM or 10
μM. Following 5 hr of LPS stimulation, supernatants
were taken and TNF-α and IL-8 levels were determined
by ELISA. In addition, cells treated with PBS or
polypeptides, but not incubated with LPS, were removed
at the time of supernatant removal to determine the
viability of the cells following 7 hr of contact with
the polypeptides. From the data depicted in **FIG. 2**,
it can be seen that the SV40 polypeptide had no effect
on TNF-α production but that the SV40MEM polypeptide
inhibited TNF-α production in a dose-dependent manner.
Similar results were obtained with respect to IL-8
production (data not shown).

<u>Example 3</u>

<u>Comparison of the Inhibition by the SV40MEM</u>

<u>Polypeptide and other Inhibitory Polypeptides</u>

<u>of κ Ig Light Chain Expression</u>

This experiment was conducted to compare the
inhibitory effect of the SV40MEM polypeptide with that
of the polypeptide AAVALLPAVLLALLAPVQRKRQKLMP (SEQ ID

NO:5) (the "NF-κBMEM" polypeptide) on κ light chain
expression in 70Z/3 B-cells.

      The 70Z/3 cells were pretreated for 2 hr
with between 0 and 50 μM of: (1) a peptide containing
5   only the FGF signal sequence AAVALLPAVLLALLAP (SEQ ID
NO:4) (the "MEM" peptide); (2) a polypeptide having
the FGF signal sequence and the NLS of NF-κB p50 on
the carboxy terminus of the signal sequence, namely,
the NF-κBMEM polypeptide; (3) the SV40MEM polypeptide
10   comprised of L-amino acids; or (4) the SV40MEM
polypeptide comprised of D-amino acids.   The cells
were then incubated with LPS as described in Example 2
and assayed for expression of κ light chain
expression.

15   The data in **FIG. 3** show that the L- and D-
amino acid forms of the SV40MEM polypeptide were
equally potent and efficacious with respect to
inhibiting LPS-stimulated κ Ig expression.   In
addition, the data show that both the L- and D-amino
20   acid forms of the SV40MEM polypeptide were more potent
and more efficacious that the NF-κBMEM polypeptide
having a single NLS sequence.   The EC_{50} for both the L-
and D-amino acid forms of the SV40MEM polypeptide was
0.7 μM, while that for the NF-κBMEM polypeptide having
25   a single NLS sequence was 32 μM.

<u>Example 4</u>
<u>Comparison of the Inhibition by the SV40MEM</u>
<u>Polypeptide Other Inhibitory Polypeptides</u>
30   <u>of Cytokine Production</u>
      This experiment was conducted to compare the
inhibitory effect of the SV40MEM polypeptide and other
inhibitory polypeptides on LPS induction of cytokine
production.   THP-1 control cells (ATCC 9/95 stock)
35   were harvested, washed and set up in RPMI 1640, 10%
fetal calf serum as described in Example B.   The cells
were incubated for 2 hr with PBS or (1) the FGF signal

sequence, (2) a polypeptide having the FGF signal
sequence and the NLS of NF-κB p50 on the carboxy
terminus of the signal sequence, (3) the SV40MEM
polypeptide comprised of L-amino acids, or (4) the

5   SV40MEM polypeptide comprised of D-amino acids at a
final concentration of 5 μM. The cells were then
plated into a 96-well plate with 0.1 ml/well. The
plate was then incubated for 2 hr at 37°C in a 5% CO_2
atmosphere. Following the initial incubation, media

10   or a titration of LPS was added to each of the wells
in a volume of 0.01 ml/well. Following 5 hr of LPS
stimulation, supernatants were taken and TNF-α levels
were determined by ELISA. The date depicted in **FIG.
4A** indicated that only the L- or D-amino acid forms of

15   the SV40MEM polypeptide significantly inhibited LPS-
induced TNF-α expression; i.e., the polypeptide
comprising the FGF signal sequence peptide and the NF-
κB NLS did not significantly inhibit TNF-α expression.
At high LPS concentrations, TNF-α was induced even in

20   cells that had been incubated with the L- or D-amino
acid forms of the SV40MEM polypeptide. Similar
results were obtained with LPS-induced IL-8 production
(**FIG. 4B**).

25   Example 5
      Effect of the SV40MEM Polypeptide on CD40 Expression
                    70Z/3 murine B-cells were treated with the
      SV40MEM polypeptide for 3 hr prior to activation with
      LPS. After activation with LPS, there was an

30   approximately four-fold increase in CD40 expression
      above basal levels. The SV40MEM polypeptide inhibited
      this up-regulation of CD40 (see **FIG. 5**). These
      results indicate that the SV40MEM polypeptide should
      be efficacious at inhibiting B-cell responses *in vivo*.

35

Example 6
Inhibition of Infection of Peripheral Blood
Mononuclear Cells with the Primary Isolate
of HIV-1 M1 by the HIV-1MEM Polypeptide

5   PBMCs were obtained washed, cultured, and
infected with HIV-1 as described in Dubrovsky et al.
(1995) *Mol. Med.* 1:217-230). PBMCs from a
seronegative donor were collected and depleted of CD8·
T cells by negative selection. The cells were
10   activated with an anti-CD3 monoclonal antibody
prepared according to the method described in Wee et
al. (1993) *J. Exp. Med.* 177:219 and incubated for 3 hr
with the HIV-1MEM polypeptide. The cells were then
incubated with M1 virus isolated from PBMC collected
15   from HIV-infected donors in the presence of inhibitors
for 2 hr. The virus was washed out, and the cultures
supplemented with the appropriate inhibitor and
allowed to incubate for seven days. Virus production
was determined by measuring the p24 levels in the
20   culture supernatant (**FIG. 6B**) and contrasted with the
effect of the polypeptide on cell proliferation (**FIG.
6A**), which virus load was determined by polymerase
chain reaction analysis of proviral DNA content (gag)
(**FIG. 6C**). The MEM peptide represents the FGF
25   membrane translocating portion of the HIV-1MEM
polypeptide. The HIV-1MEM polypeptide significantly
reduced the virus load and virus production compared
to untreated but activated cultures.

30   Example 7
Inhibition of Infection of T Cells
with HIV$_{M1}$ Virus by the HIV-1MEM Polypeptide

        H9 and Jurkat cells were incubated with the
HIV-1MEM polypeptide (2 μM) or the control MEM peptide
35   for 4 hr prior to addition of HIV-M1 virus. Cells
were harvested on day 3 following injection and the
level of HIV-1 DNA evaluated by polymerase chain

reaction. As shown in **FIG. 7**, the HIV-1MEM polypeptide inhibited infection of both cells lines as determined by the lower levels of HIV-1 DNA compared to untreated and MEM peptide-treated cultures.

5

Example 8
Reduction in Viral Infection Due to
Reduction in Nuclear Translocation of HIV Genome

Jurkat cells were incubated with the SV40MEM
10   polypeptide for 3 hr with HIV$_{LAI}$ for 1-2 hr. The virus was washed out and the cultures supplemented with the appropriate inhibitor (SV40MEM, 0.1 μM and 1.0 μM; NF-κBMEM, 10 μM). After 24 hr, the cells were collected, lysed and evaluated for HIV-1 DNA by polymerase chain
15   reaction analysis of gag sequences (see **FIG. 8A**) and 2-LTR circles (see **FIG. 8B**). The uniform signal achieved in all samples when assayed for gag sequences shows that all cultures internalized virus relatively equally and allowed formation of the viral cDNA in the
20   cytoplasm. The difference in the levels of 2-LTR circles shows that SV40MEM inhibited translocation of the viral genome into the nucleus. Thus, the polypeptides do not affect virus entry into cells but, as predicted, specifically target nuclear
25   translocation. The NF-κBMEM peptide showed no effect on viral internalization or translocation of the viral genome into the nucleus.

Example 9
30   ### Effect of SV40MEM on Proliferation
of 70Z/3 Murine B-cell Leukemia Cells

Murine 70Z/3 B-cells were treated with 0, 0.2, 0.5, 1, or 2 μM SV40MEM prepared from D-amino acids. On day 0, the cell concentrations of the
35   control and treated cells were the same. After 48 hr of exposure to the peptide, the treated cells were again counted and compared to control cells. **FIG. 9**

shows that the proliferation of the murine leukemia cell line was inhibited by the lowest concentration (0.2 μM) of D-SV40MEM tested and was 90% inhibited by the highest concentration tested (2 μM).

5

Example 10

Effect of SV40MEM on Proliferation
of RAJI Human B-cell Leukemia Cells

Human RAJI B-cells were treated with 0, 5, 10 or 25 μM SV40MEM prepared from D-amino acids. On day 0, the cell concentrations of the control and treated cells were the same. After 48 hr of exposure to the peptide, the treated cells were again counted and compared to control cells. **FIG. 10** shows that the proliferation of the murine leukemia cell line was inhibited by the lowest concentration (5 μM) of D-SV40MEM tested and was greater than 95% inhibited by the highest concentration tested (25 μM).

20

Example 11

In Vivo Immunosuppressive Effect of the SV40MEM
Polypeptide In a Murine Sheep Red Blood Cell Assay

Female BALB/c mice (6-8 weeks of age) were housed in groups of 5 in Thorne units with *ad libitum* access to food and water. Mice were immunized on day 0 by intravenous (IV) injection of 1 x 10^8 sheep red blood cells ("SRBCs"). The mice were then divided into groups of five and control solutions or inhibitory peptides were administered at various doses, either by IV injection or orally ("PO") by gavage with a 24-gauge feeding tube and routes.

The SV40MEM polypeptide was administered either intravenously (IV) or orally (PO) with the following schedule: IV administration of 5 mg/kg on days 0, 1, 2, and 3; PO administration of 1 mg/kg on days 0, 1, 2, 3, and 4. After 14 days, the mice were bled from the orbital sinus and the level of anti-SRBC

antibodies were measured in the serum by ELISA.
Briefly, Immulon 2 plates (Dynatech) were coated with
SRBC membrane antigen (*Anal. Biochem.* 82:362-371
(1978)) at 5 μg/ml in 0.05 M carbonate/bicarbonate
5   buffer (pH 9.6) and incubated overnight at 2-8°C.
Plates were blocked for 1-hr at room temperature with
Specimen Diluent Concentrate (Genetic Systems,
Redmond, WA) diluted 1:10 with distilled water. The
plates were washed in phosphate-buffered saline and
10   0.05% Tween-20 ("PBS/Tween"). Serum samples were
diluted in Specimen Diluent and incubated for 1 hr at
room temperature. After washing the plates in
PBS/Tween, goat anti-mouse IgG1-horse radish
peroxidase-conjugated ("IgG1-HRP") antibody (Southern
15   Biotechnology, Birmingham AL) was added at 1:5000
dilution and incubated for 1 hr at room temperature.
Plates were washed and conjugate binding was detected
using 3,3',5,5'-tetramethylbenzidine ("TMB") (Genetic
Systems) as the substrate. TMB was added and
20   incubated for 15 min at room temperature. The
reaction was stopped by the addition of 3 N H_2SO_4.
Absorbance at 45 nm was recorded from duplicate
samples using a microtiter plate reader (Bio Tek
Instruments).

25   The results presented in **FIG. 11A** and **FIG.
11B** show that, while there was no effect on total IgG
levels in the mice receiving the SV40MEM polypeptide
either IV or PO, the polypeptide inhibited the
specific anti-SRBC response when administered either
30   IV or PO.

Example 12
Comparison of the Inhibition by the BMS 205820
Polypeptide and other Inhibitory Polypeptides
35   of the Anti-Hemocyanin Response in Mice
Female BALB/c mice (8 weeks of age) were
immunized by intraperitoneal injection of 25 μg

keyhole limpet (*Megathura crenulata*) hemocyanin
("KLH") (Pacific Bio-Marine Laboratories, Venice, CA)
without adjuvant on day 0. Mice were divided into
groups of five and inhibitory peptides C-MYCMEM and

5   BMS 205820 (PKKKRKVAAVALLPAVLLALLAPKKKRKV (SEQ ID
NO:24)), which is identical to the SV40MEM polypeptide
with the exception that the polypeptide lacks the C-
terminal cysteine, were administered on day 0 at 5
mg/kg, followed by 5 subsequent doses of the same

10 concentration every other day. Control mice were
treated with a single 200-μg dose of Chimeric L6
antibody ("Chi L6"). After 14 days, the mice were
bled from the orbital sinus and the level of anti-KLH
antibodies were measured in the serum by ELISA as

15 described in Example 11.
        Both the BMS 205820 and the C-MYCMEM
polypeptide inhibited the specific anti-KLH response
(*see* **FIG. 12**).

20   Example 13
              Stimulation of Apoptosis by the SV40MEM
          Polypeptide in Anti-CD3-treated Jurkat T-Cells
        A 24-hr incubation of Jurkat T-cells with
SV40MEM and anti-CD3, caused greater than 85% of the

25 cells to apoptose. Neither SV40MEM nor anti-CD3 alone
caused significant apoptosis.

                            Example 14
                  Inhibition of IκB Degradation

30   by the SV40MEM Polypeptide
        IκB is an inhibitor of NF-κB nuclear
translocation. When cells are activated by the
appropriate proinflammatory stimulus, e.g., cytokines
or LPS, IκB is degraded. This results in the

35 unmasking of the NLS of NF-κB, thereby allowing NF-κB
to be translocated into the nucleus. This experiment

was done to explore the effect of the SV40MEM polypeptide on IκB degradation in murine 70Z/3 cells.

Murine 70Z/3 cells (5 x 10⁷ cells) were incubated with the SV40MEM polypeptide (2 μM) or PBS. The two groups of cells were subdivided into aliquots and activated with LPS (100 ng/ml) for 0, 15, 30, 60, or 120 min. The cells were lysed in 200 μL lysing buffer (20 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) ("HEPES"), pH 7.2, 20 mM NaCl, 2.5 mM MgCl₂, 0.1% nonylphenoxy polyethoxy ethanol ("NP-40")). The lysates were frozen until analyzed for the presence of IκB by Western analysis using an antibody directed to the carboxy terminus of IκB (Santa Cruz Corp., Santa Cruz, CA) according to the manufacturer's instructions.

Degradation of IκB was apparent after 15-min activation of the control cells with LPS. There was no apparent degradation of IκB in cells pretreated with the SV40MEM peptide.

Example 15
Ability of BMS 205820 to Treat Septic Shock

These experiments show that BMS 205820 is efficacious for the treatment of septic shock. A lethal model of septic shock was used as follows.

A. Balb/c mice were pretreated with either 5 mg/kg BMS 205820 or PBS, intravenously at 1 and 3 hours, prior to injection with 200 mg lipopolysaccharide (LPS), the causative agent of septic shock. LPS was administered intraperitoneally. Mice were given another injection of the polypeptide immediately after the LPS injection, as well as 24 hours after the LPS injection. Mice typically died within 48 hours of the LPS injection. As can be seen in Table 1, in two separate experiments, administration of BMS 205820 significantly increased

the number of mice surviving seven days post- LPS injection.

Table 1

Fraction of Mice Surviving Seven
Days Post LPS Injection

Experiment	LPS + PBS	LPS + BMS 205820
1	0/10	6/10
2	2/10	10/10

B. Balb/c were treated with 200 μg of LPS followed by the BMS 205820 polypeptide, at the times indicated in Table 2. The BMS polypeptide was administered intravenously and the LPS was administered intraperitoneally. Group A mice were treated with 5 mg/kg of polypeptide in PBS at the indicated times, post- LPS administration. Group B mice received an additional dose of polypeptide 3 hours post-LPS treatment.

As can be seen in Table 2, BMS 205820 was effective in inhibiting death in response to LPS, especially when two doses of polypeptide were administered.

Table 2

Effect of Delayed Treatment with BMS 205820
in a Lethal Model of Septic Shock

Treatment	Fraction Surviving	
	Group A	Group B
None	0/5	2/5
T=0	5/5	5/5
T=30 min.	1/5	5/5
T=1 hr.	2/5	5/5
T=1.5 hr.	1/5	4/5
T=2 hr.	0/5	0/5

Example 16

Inhibition of Cytokine Production *in vivo*
by the BMS 205820 Polypeptide

These experiments show that the BMS 205820
polypeptide is capable of modulating cytokine
production. In particular, a non-lethal model of
septic shock was used. Balb/c mice were treated with
1 μg LPS, and at the same time, a single dose of 3
mg/kg of the BMS 205820 polypeptide in PBS. TNF-α,
IL-6 and IL-10 levels were measured in serum by ELISA.

As shown in **FIG. 13A**, the BMS 205820
polypeptide caused a significant inhibition in the
production of TNF-α, which is a major contributor to
the sepsis syndrome. IL-6 levels were only slightly
affected (**FIG. 13B**), whereas IL-10 production was
greatly increased (**FIG. 13C**). The effect on IL-10 is
beneficial since this cytokine is immunosuppressive
and has been shown to have efficacy in models of
septic shock.

Example 17

Inhibition of LPS Binding to CD14 Using
BMS 205820 and C-MYCMEM

These experiments show that BMS 205820 and
C-MYCMEM (the "BMS 214572" polypeptide) are both
capable of inhibiting the binding of LPS to its cell
surface receptor, CD14, evidencing that the
polypeptide inhibitors are useful in the treatment of
sepsis.

96-well plates were coated with goat-anti
human Ig, soluble CD-14 Ig was added, and the plates
were incubated overnight at 4°C. The BMS polypeptide,
the C-MYCMEM polypeptide, an SV40 NLS polypeptide
containing a single SV40 large T-antigen NLS, a c-myc
NLS without a translocation sequence (AKRVKL (SEQ ID
NO:6)), a control non-NLS polypeptide, 377G, or PBS,
were added to the plates and the plates were incubated

-40-

for 5 min. *E. coli* AO16 LPS was then added to the
plates and the plates were incubated for 2 hours at
4°C. The plates were washed and LPS was detected
using 0.5 μg/ml mouse anti-*E. coli* LPS, followed by
5   goat anti-mouse horseradish peroxidase.

FIG. 14 shows the results of the experiment.
Both BMS 205820 and C-MYCMEM were able to bind to LPS
and block it from binding CD14. The c-myc NLS did not
block binding of LPS to CD14.

10   LPS is the causative agent of septic shock.
Thus, by blocking binding of LPS to CD14, the NLS
polypeptides described herein may serve to prevent or
diminish septic shock *in vivo*. This result was
unexpected. Without being bound by a particular
15   theory, it is possible that positively charged NLS
polypeptides bind to negatively charged regions on
LPS.

Example 18
20   Ability of BMS 205820 to Treat Asthma

These experiments show that the BMS 205820
polypeptide is efficacious for the treatment of
asthma. The BMS 205820 polypeptide was tested in a
mouse model of asthma (Renz et al. (1992) *J. Allergy*
25   *Clin. Path.* 89:1127-1138). Balb/c mice were
administered ovalbumin in an alum adjuvant
intraperitoneally at various times, as indicated in
FIG. 15A. Treatment was followed by nebulization of
ovalbumin into the lungs, also as indicated in FIG.
30   15A. The mice were also treated with 3 mg/kg of the
BMS 205820 polypeptide in PBS at the times indicated.
Mice were sacrificed at day 18 and the presence of
eosinophils assayed. As seen in FIG. 15B, the BMS
205820 polypeptide significantly inhibited the
35   infiltration of eosinophils into the lungs, a measure
of the asthmatic state.

Thus, inhibitors of nuclear translocation of cellular proteins have been disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that

5   obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

10

15

20

25

30

35

WE CLAIM:

        1.   An isolated polypeptide comprising:

        (1) a signal sequence peptide capable of
5   delivering the polypeptide through the cytoplasmic
membrane into a cell; and

        (2) at least two nuclear localization
sequences (NLSs),

        wherein said polypeptide is capable of
10   inhibiting nuclear translocation of a cellular
protein.

        2.   The polypeptide of claim 1, wherein the
signal sequence is interchangeably flanked at its
15   amino- and carboxy-termini by first and second NLSs.

        3.   The polypeptide of either of claims 1
or 2, wherein the signal sequence peptide is the
antennapedia homeodomain signal sequence peptide, the
20   fibroblast growth factor signal sequence peptide, the
human immunodeficiency virus (HIV) Tat signal sequence
peptide, or the Hsc70 signal sequence peptide.

        4.   The polypeptide of claim 3, wherein the
25   signal sequence peptide is the antennapedia
homeodomain signal sequence peptide.

        5.   The polypeptide of claim 4, wherein the
signal sequence peptide comprises the amino acid
30   sequence RQIKIWFQNRRMKWKK (SEQ ID NO:7).

        6.   The polypeptide of claim 3, wherein the
signal sequence peptide is the fibroblast growth
factor signal sequence peptide.

35

-43-

7. The polypeptide of claim 6, wherein the signal sequence peptide comprises the amino acid sequence AAVALLPAVLLALLA (SEQ ID NO:8).

5 8. The polypeptide of claim 3, wherein the signal sequence peptide is the HIV Tat signal sequence peptide.

9. The polypeptide of claim 8, wherein the
10 signal sequence peptide comprises the amino acid sequence CFITKALGISYGRKKRRQRRRPPQGSQTH (SEQ ID NO:9).

10. The polypeptide of either of claims 1 or 2, wherein the first and second NLSs may be the
15 same or different and are peptides comprising the amino acid sequence selected from the group consisting of PKKKRKV (SEQ ID NO:10), KKKRKVC (SEQ ID NO:11), GKKRSKA (SEQ ID NO:12), KRPRP (SEQ ID NO:13), GNKAKRQRST (SEQ ID NO:14), GGAAKRVKLD (SEQ ID NO:15),
20 SALIKKKKKMAP (SEQ ID NO:16), RKLKKLGN (SEQ ID NO:17), PQPKKKP (SEQ ID NO:18), ASKSRKRKL (SEQ ID NO:19), KKKYK (SEQ ID NO:20), KKKYKC (SEQ ID NO:21), KSKKK (SEQ ID NO:22), KRVKLC (SEQ ID NO:23), and AKRVKL (SEQ ID NO:6).

25

11. The polypeptide of claim 7, wherein the first and second NLSs may be the same or different and are peptides comprising the amino acid sequence selected from the group consisting of PKKKRKV (SEQ ID
30 NO:10), KKKRKVC (SEQ ID NO:11), GKKRSKA (SEQ ID NO:12), KRPRP (SEQ ID NO:13), GNKAKRQRST (SEQ ID NO:14), GGAAKRVKLD (SEQ ID NO:15), SALIKKKKKMAP (SEQ ID NO:16), RKLKKLGN (SEQ ID NO:17), PQPKKKP (SEQ ID NO:18), ASKSRKRKL (SEQ ID NO:19), KKKYK (SEQ ID
35 NO:20), KKKYKC (SEQ ID NO:21), KSKKK (SEQ ID NO:22), KRVKLC (SEQ ID NO:23), and AKRVKL (SEQ ID NO:6).

12.   The polypeptide of claim 11, wherein the first and second NLSs comprise the amino acid sequence PKKKRKV (SEQ ID NO:10).

5   13.   The polypeptide of claim 11, wherein the first and second NLSs comprise the amino acid sequence PKKKRKV (SEQ ID NO:10) and KKKRKVC (SEQ ID NO:11), respectively, KKKYK (SEQ ID NO:20) and KKKYKC (SEQ ID NO:21), respectively, or AKRVKL (SEQ ID NO:6) 10   and KRVKLC (SEQ ID NO:23), respectively.

14.   The polypeptide of claim 12 comprising the amino acid sequence PKKKRKVAAVALLPAVLLALLAPKKKRKV (SEQ ID NO:24).

15

15.   The polypeptide of claim 13 comprising the amino acid sequence PKKKRKVAAVALLPAVLLALLAPKKKRKVC (SEQ ID NO:1).

20   16.   The polypeptide of any of claims 1-15, wherein the cellular protein is a transcription factor.

17.   The polypeptide of claim 16, wherein 25   the transcription factor is NF-κB.

18.   A method of introducing an exogenous polypeptide comprising a nuclear localization sequence (NLS) into an intact cell so as to inhibit nuclear 30   translocation of a cellular protein, comprising:
          (a)   providing a polypeptide according to any of claims 1-17; and
          (b)   contacting the cell with the polypeptide for a period of time effective to 35   introduce the exogenous polypeptide into the cell.

19.   A method of suppressing an immune response in a subject comprising administering to the subject an immunosuppressive amount of a polypeptide according to any of claims 1-17.

5

20.   A method of treating or preventing a viral infection in a subject, comprising administering to the individual an effective antiviral amount of a polypeptide according to any of claims 1-17.

10

21.   A method of transcriptionally modulating the expression of cellular genes comprising, contacting a target cell with an inhibitor of nuclear translocation of a cellular protein,

15   wherein the cellular protein is a transcription factor, and wherein the inhibitor comprises a polypeptide according to any of claims 1-17.

22.   Use of a polypeptide according to any

20   of claims 1-17 for the manufacture of a composition useful for suppressing an immune response in a subject.

23.   Use of a polypeptide according to any

25   of claims 1-17 for the manufacture of a composition useful for treating or preventing a viral infection in a subject.

24.   Use of a polypeptide according to any

30   of claims 1-17 for the manufacture of a composition useful for transcriptionally modulating the expression of cellular genes.

35
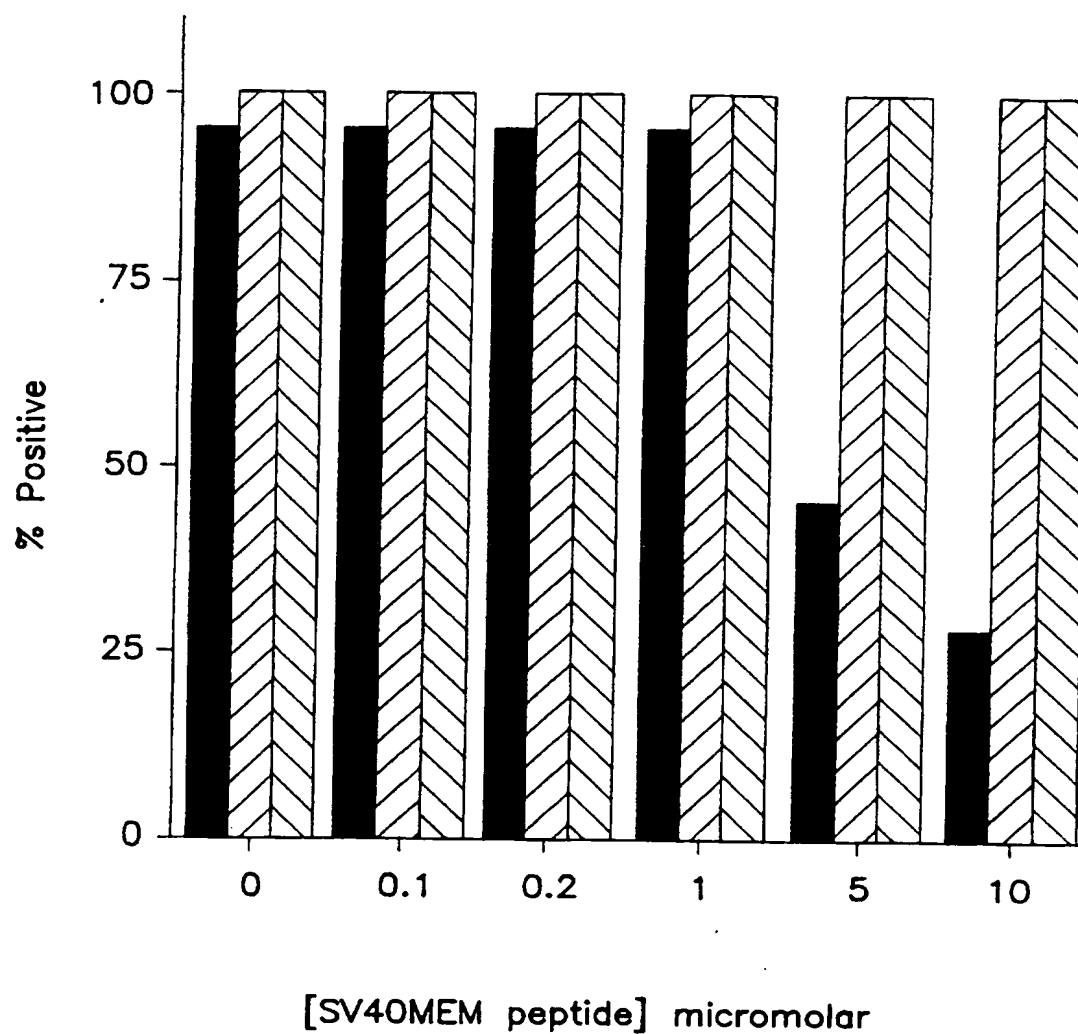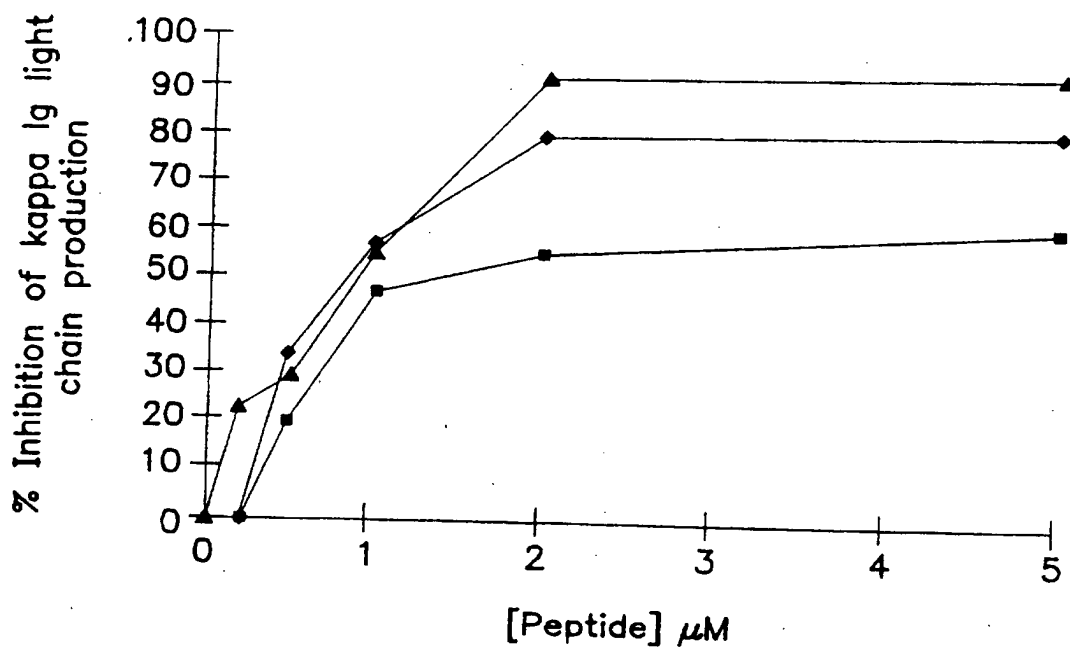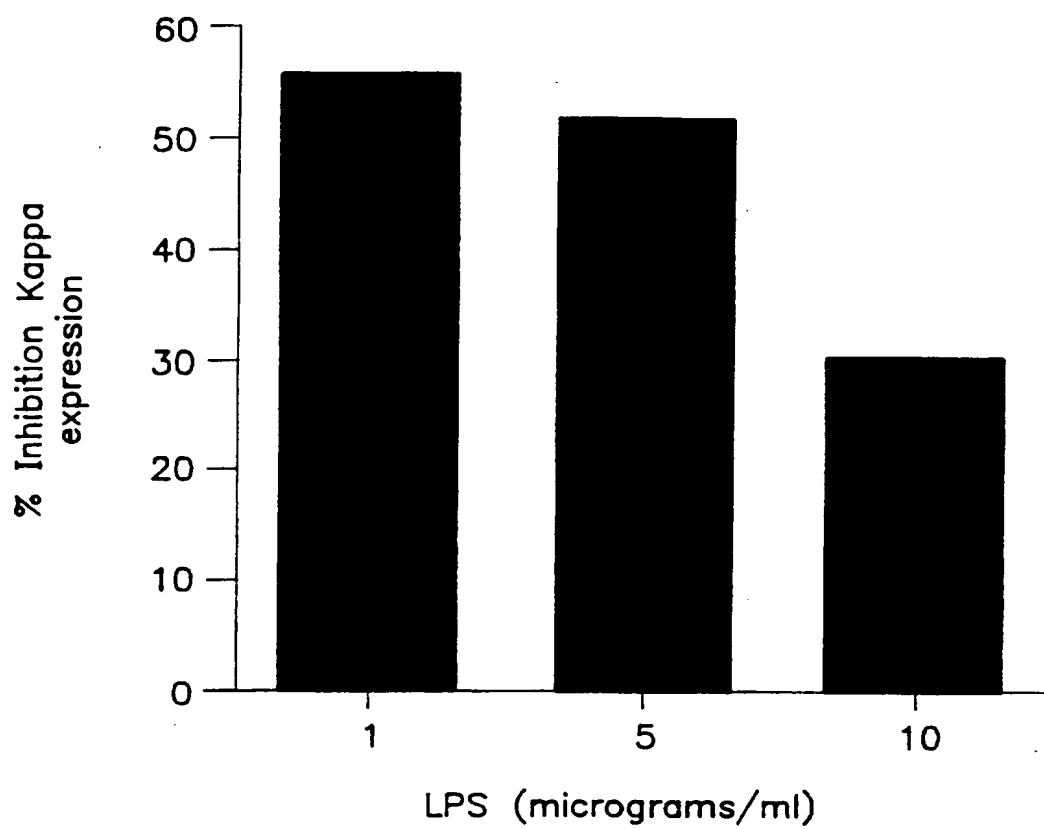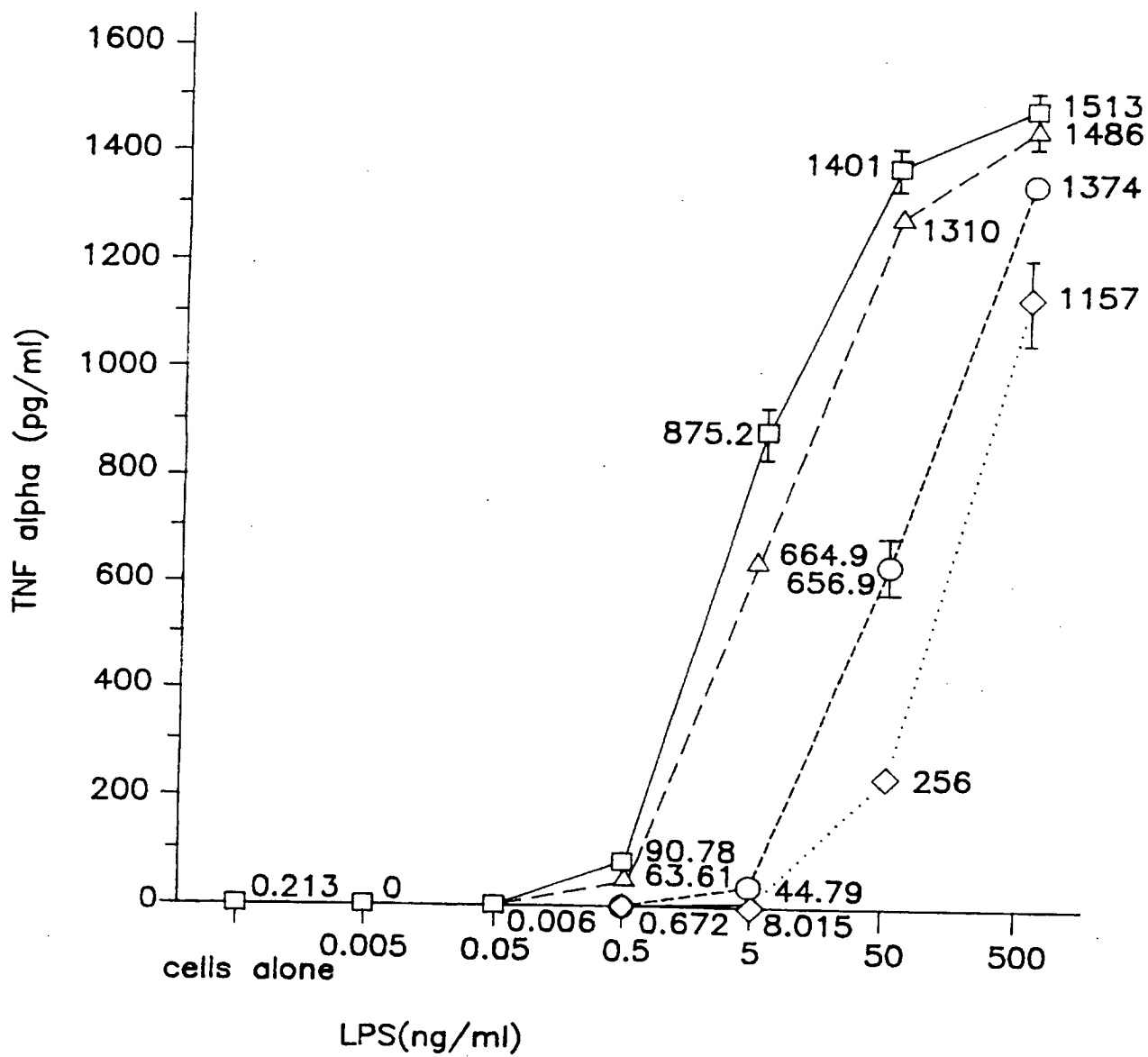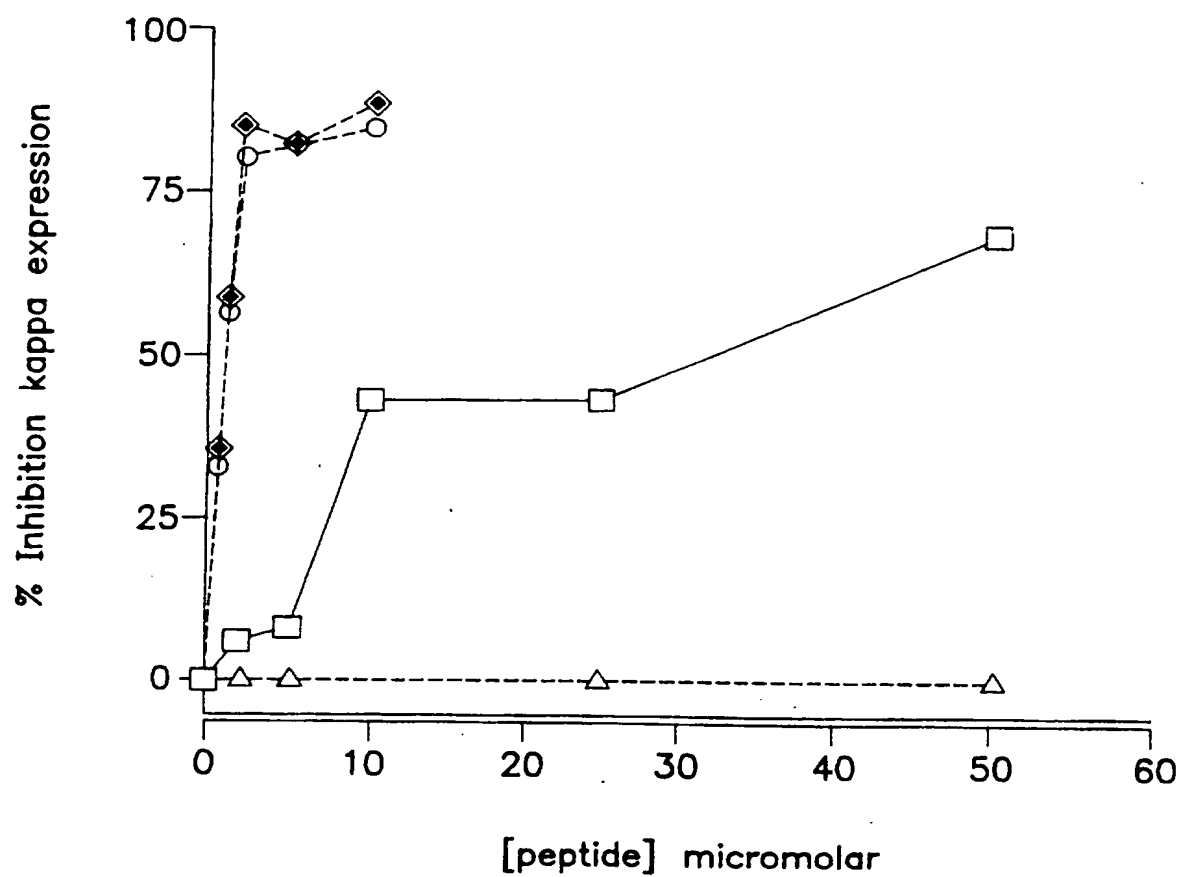
[SV40MEM peptide] micromolar

FIG. IA

FIG. IB

FIG. IC

FIG. 2

FIG. 3

FIG. 4A

FIG. 4B

FIG. 5A

FIG. 5B

FIG. 6A

FIG. 6B

● ●

unactivated

HIV-1 MEM
peptide (μM)

MEM peptide

| 0 | 10 | 1 | 10 |

FIG. 6C

untreated

HIV-1 MEM peptide (μM)

MEM peptide

H9

Jurkat
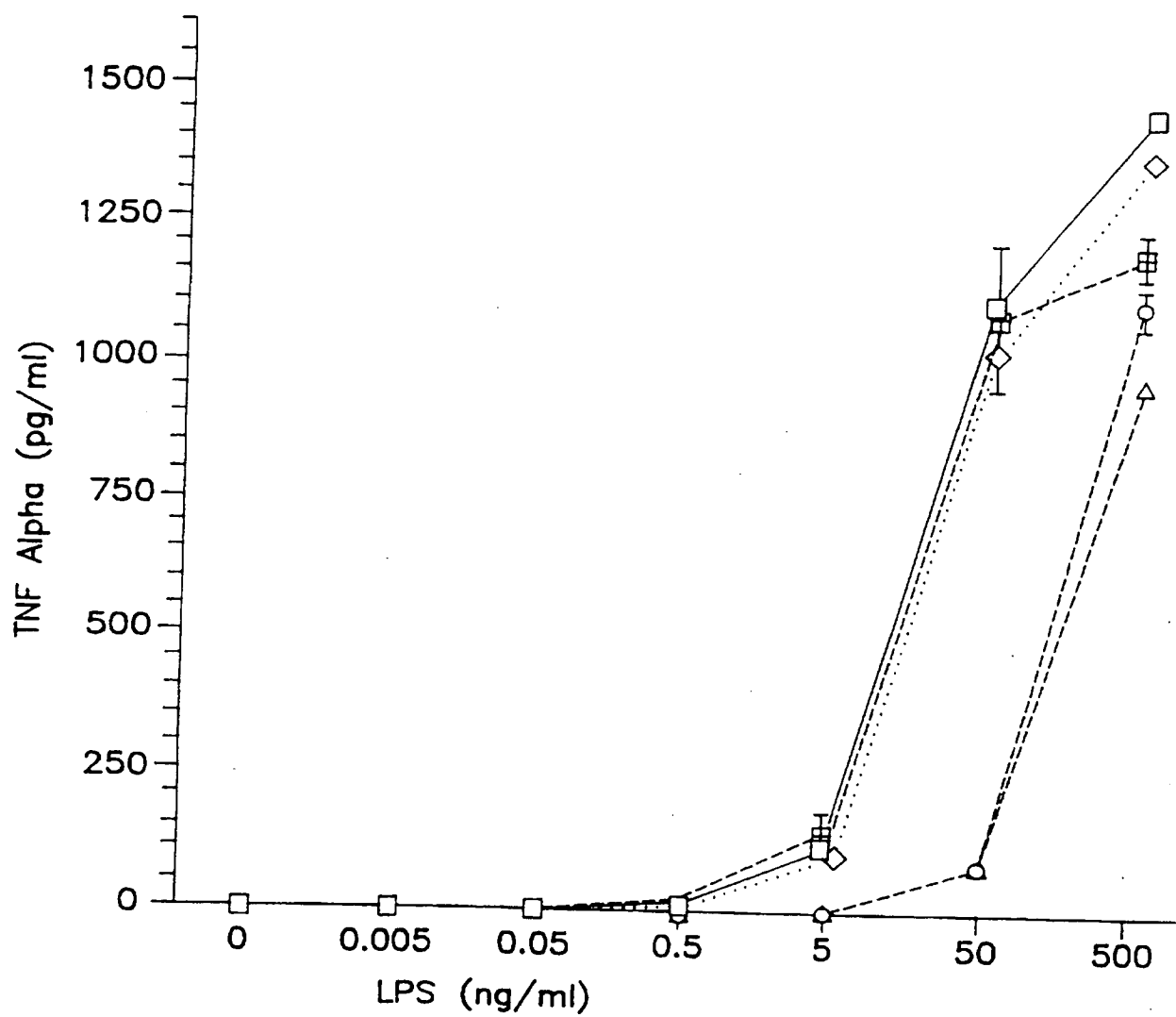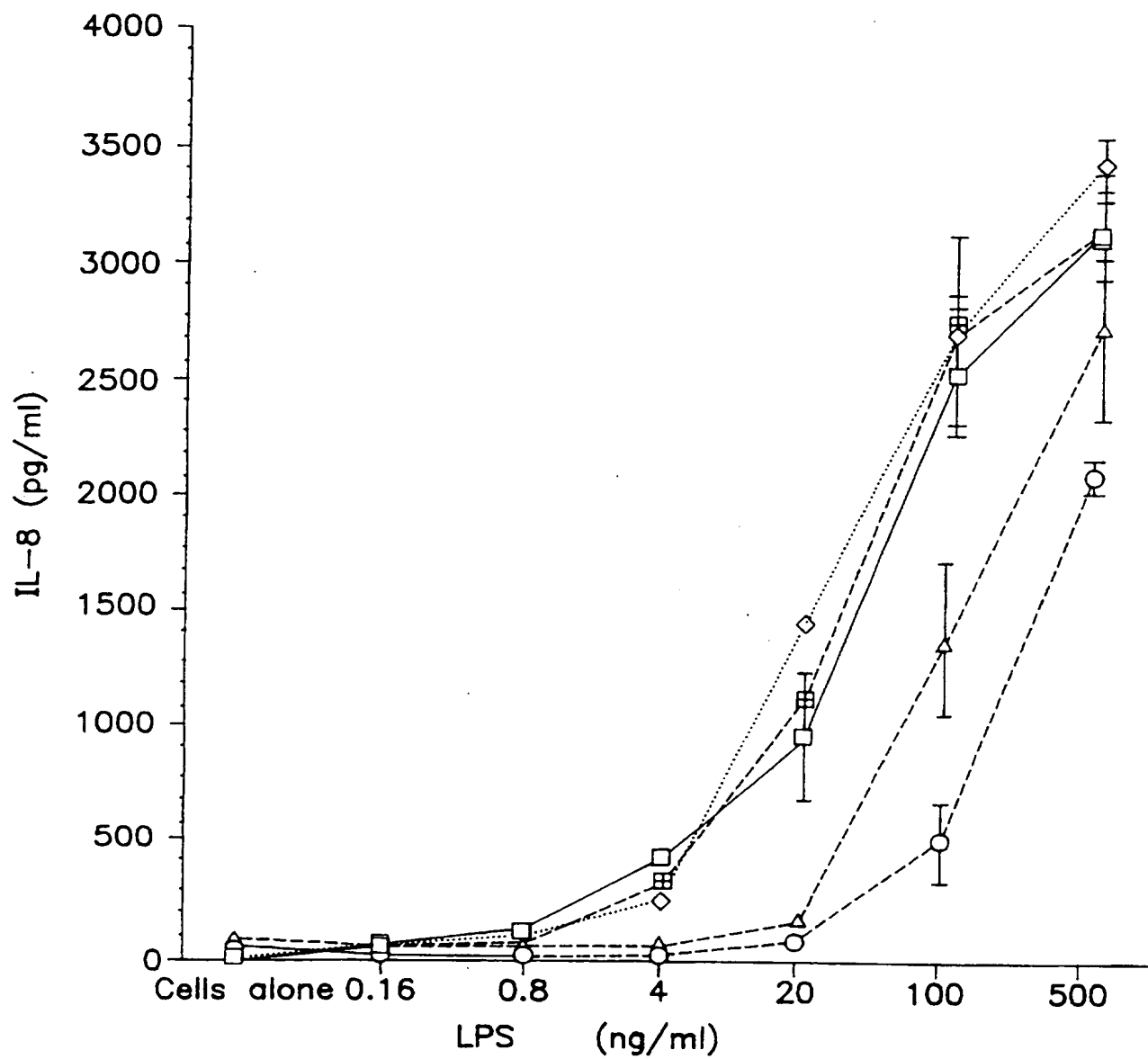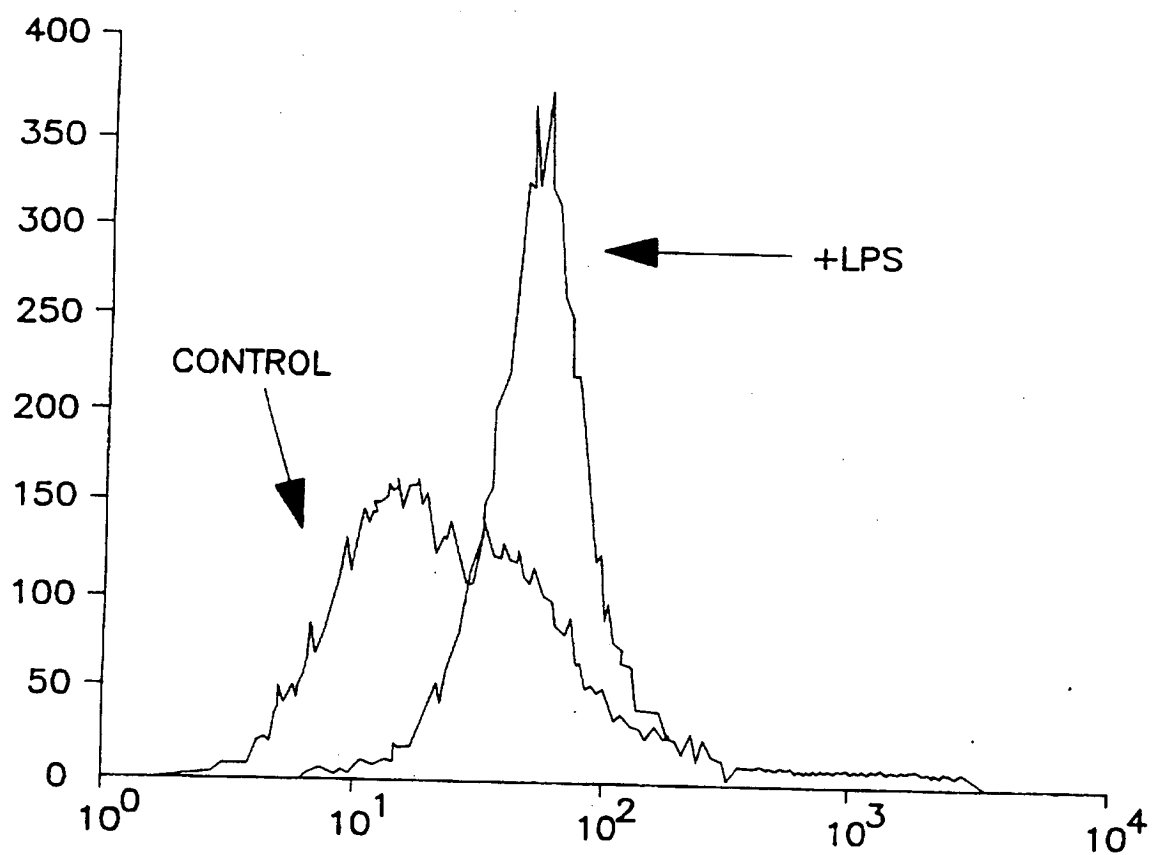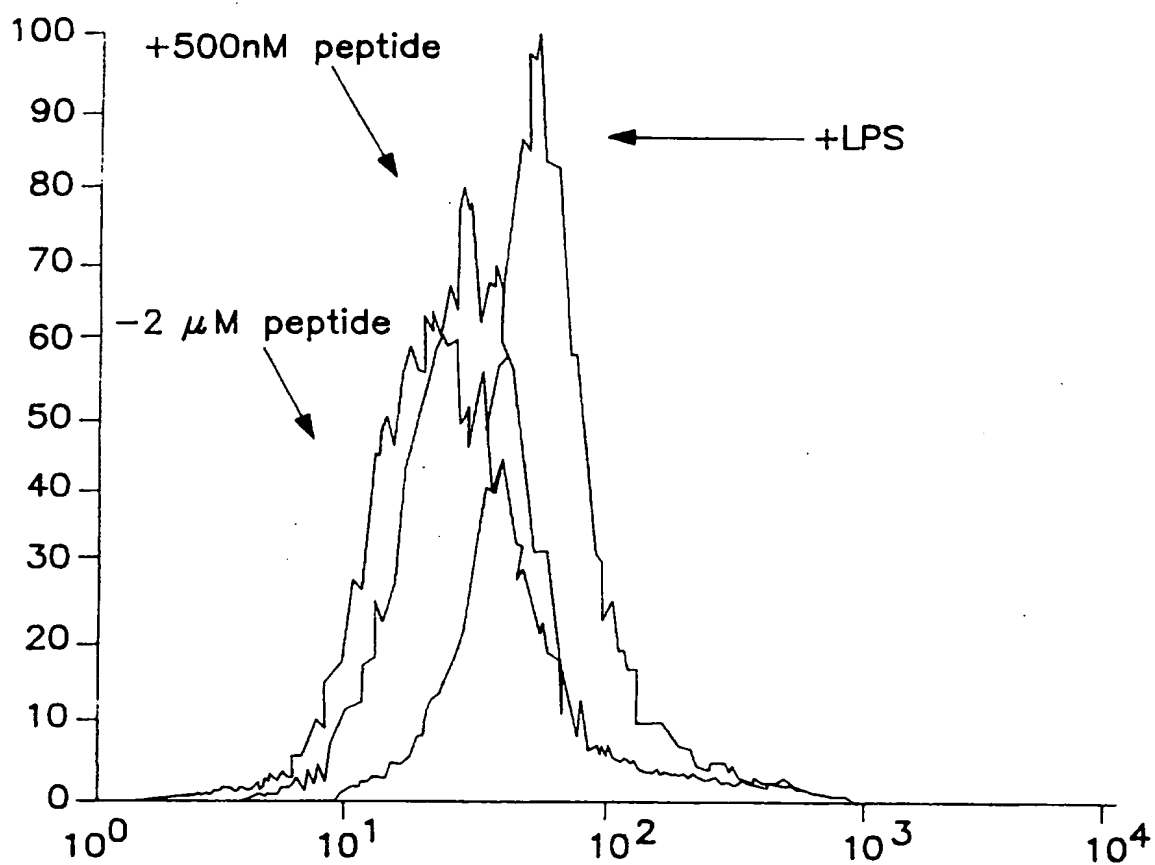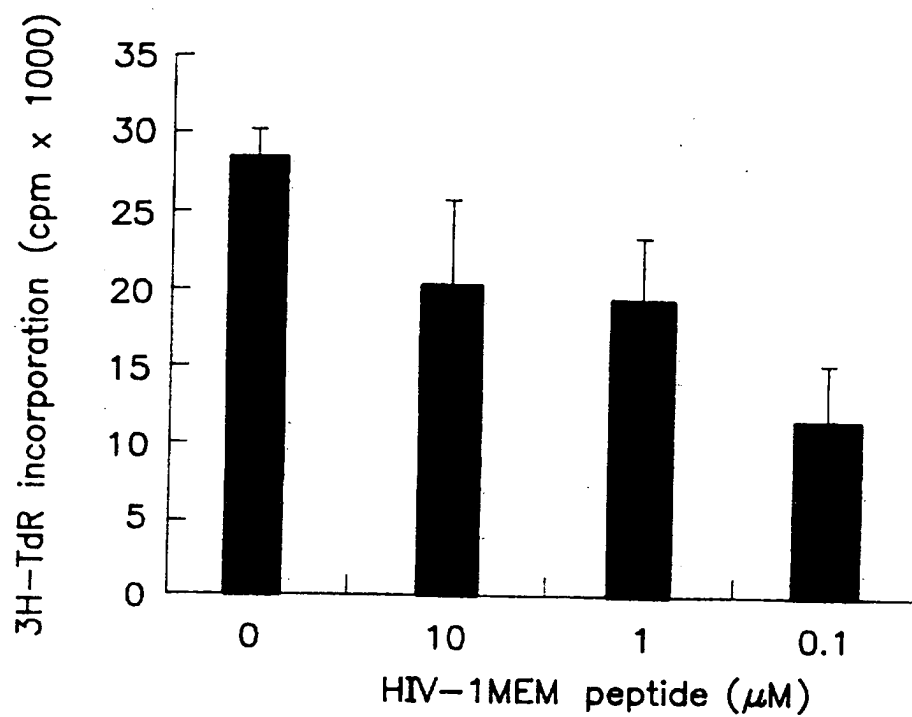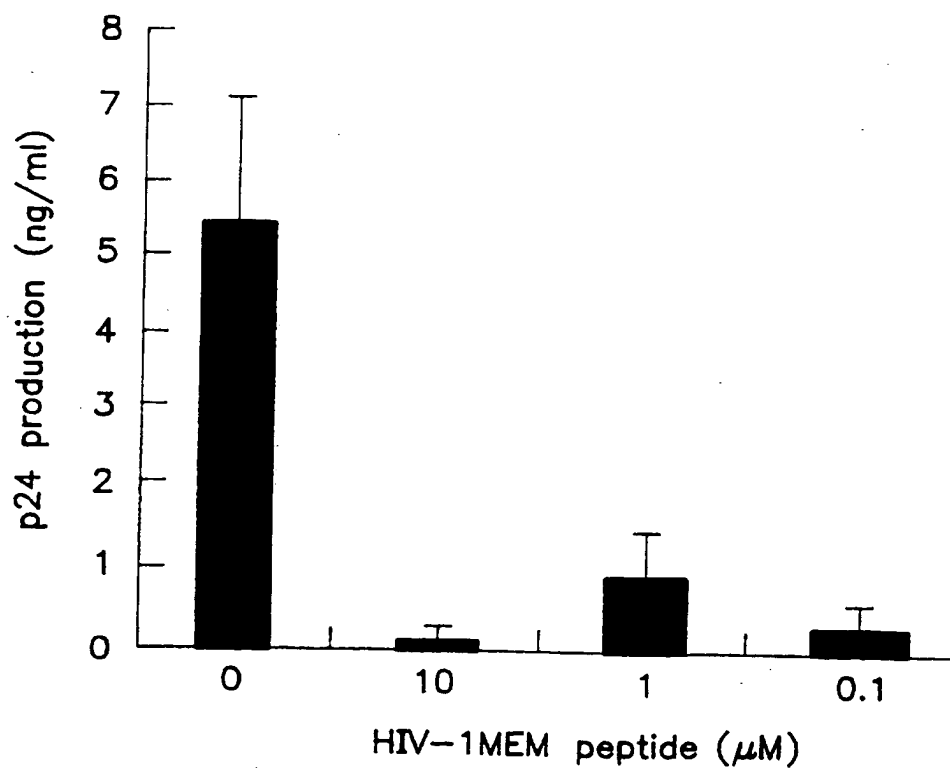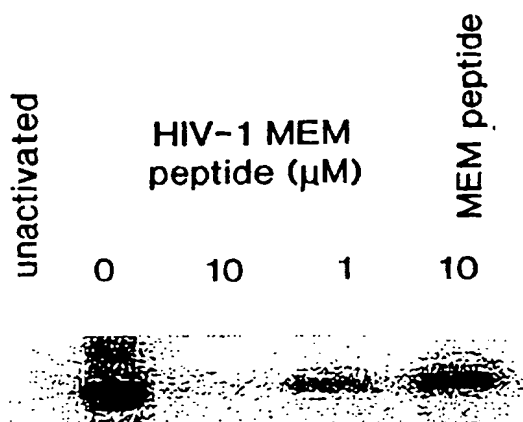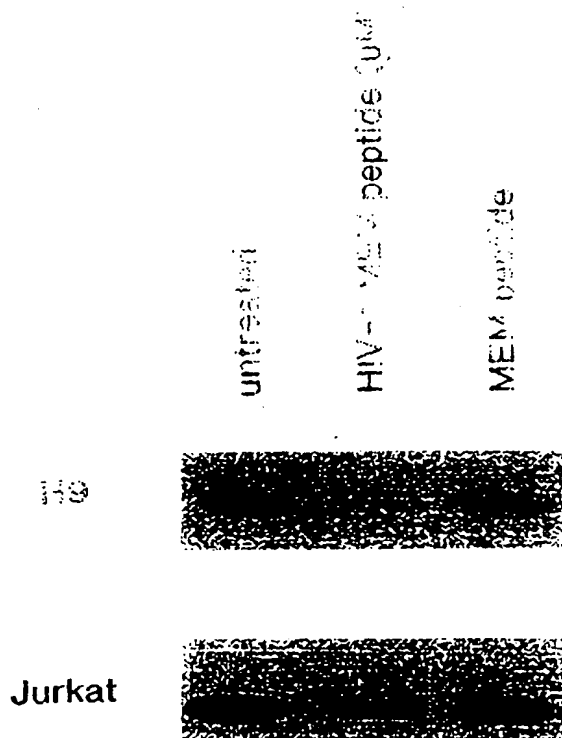
FIG. 7

SV40MEM peptide

NF-κBMEM peptide

FIG. 8A

FIG. 8B
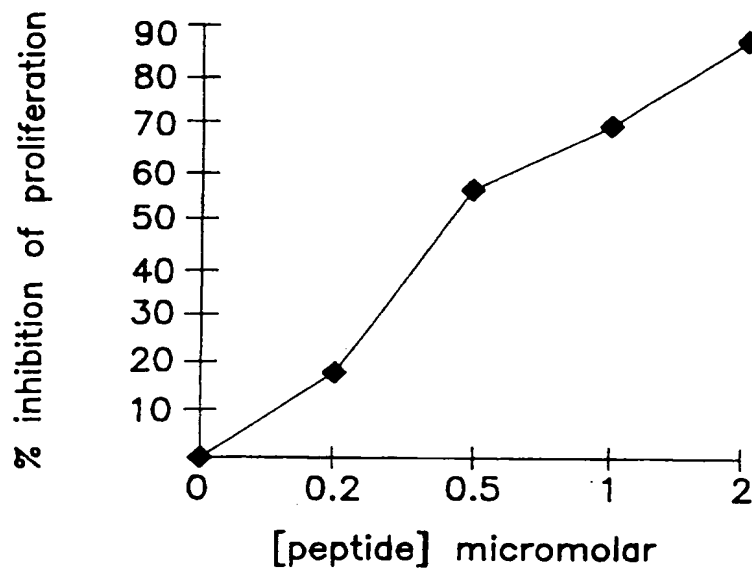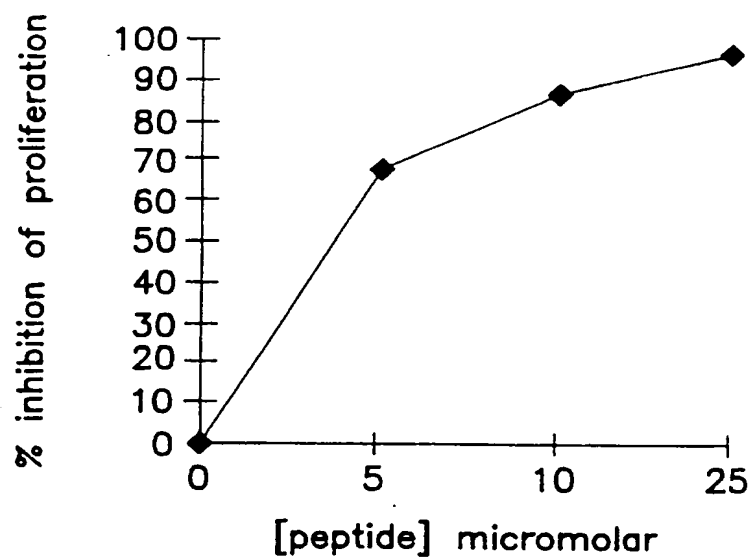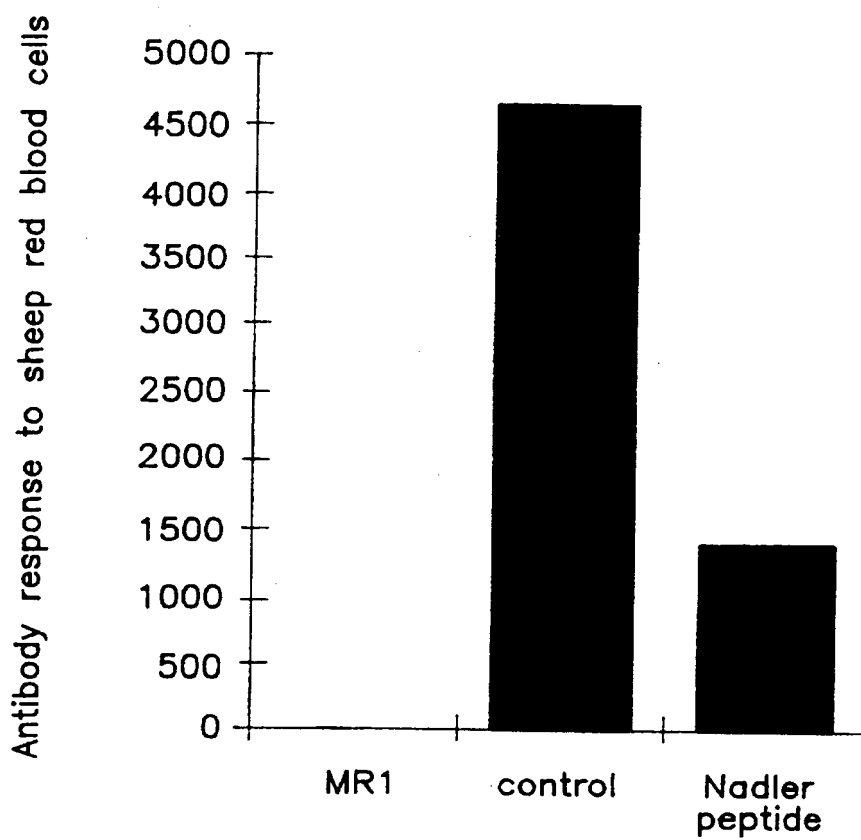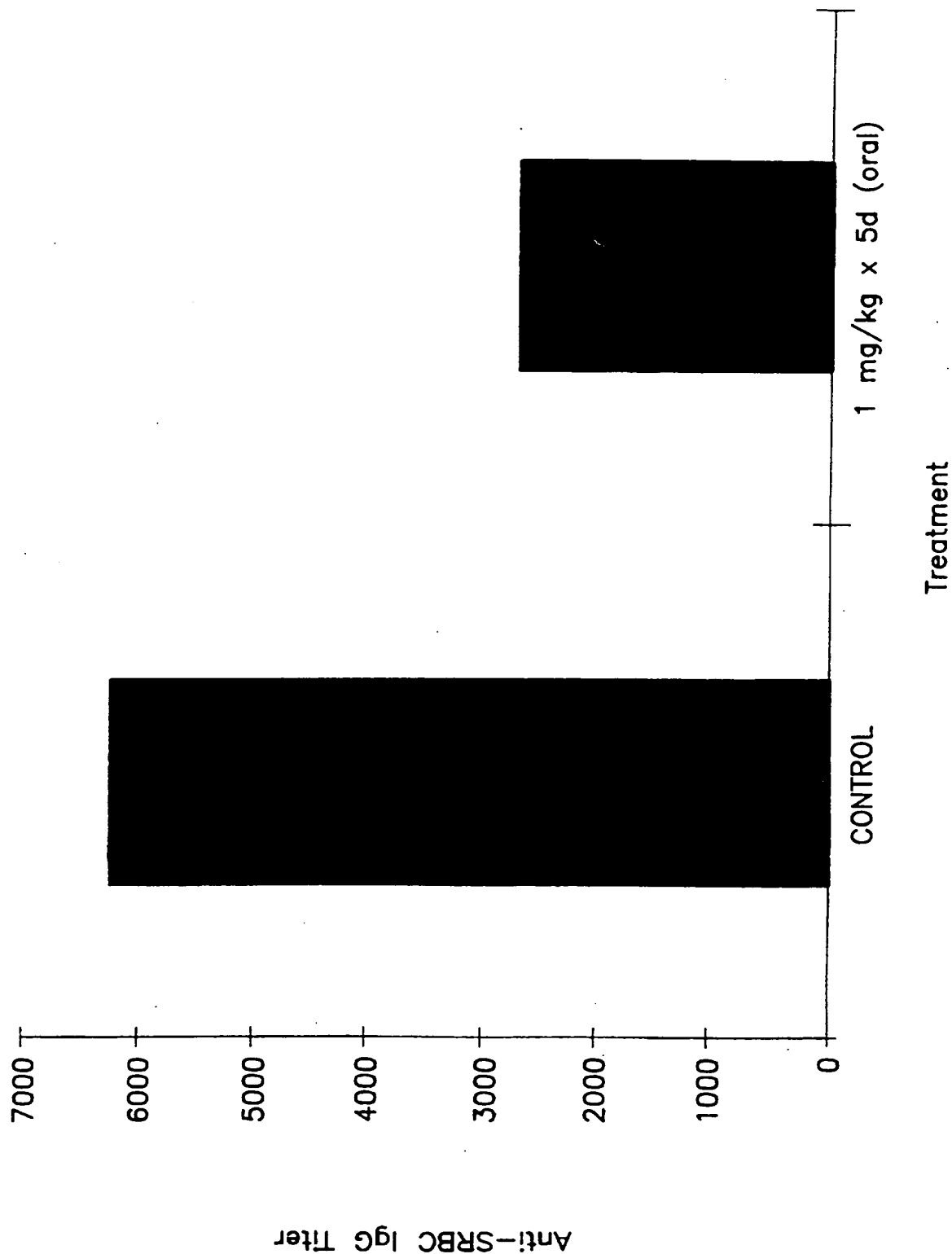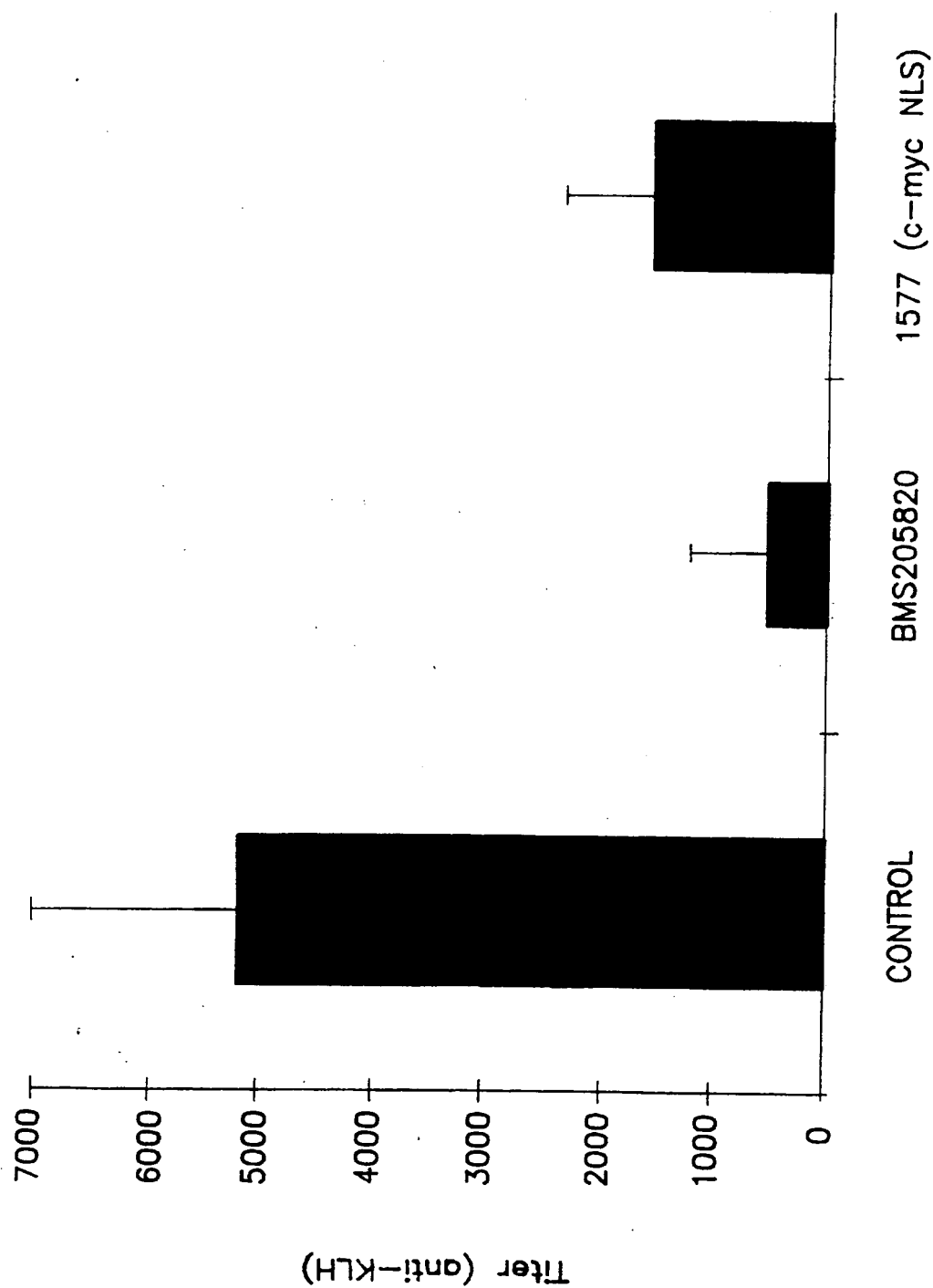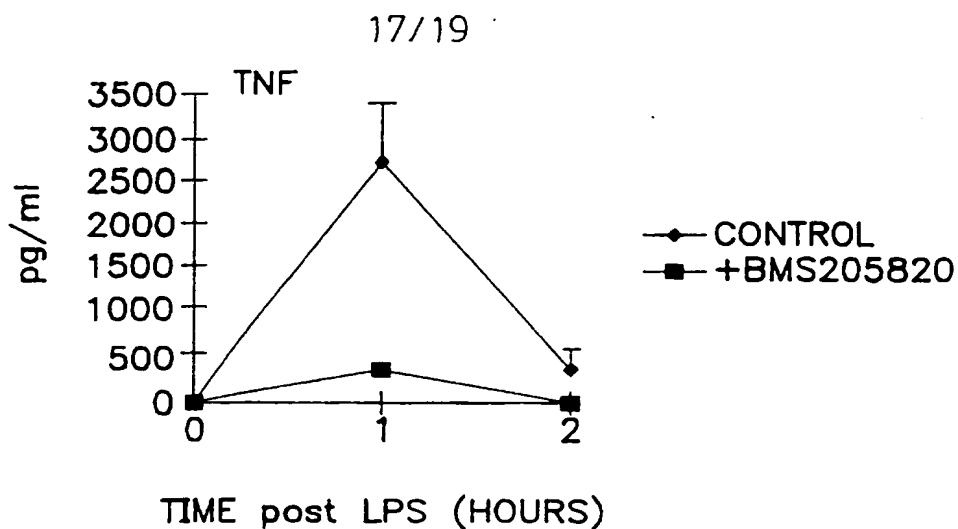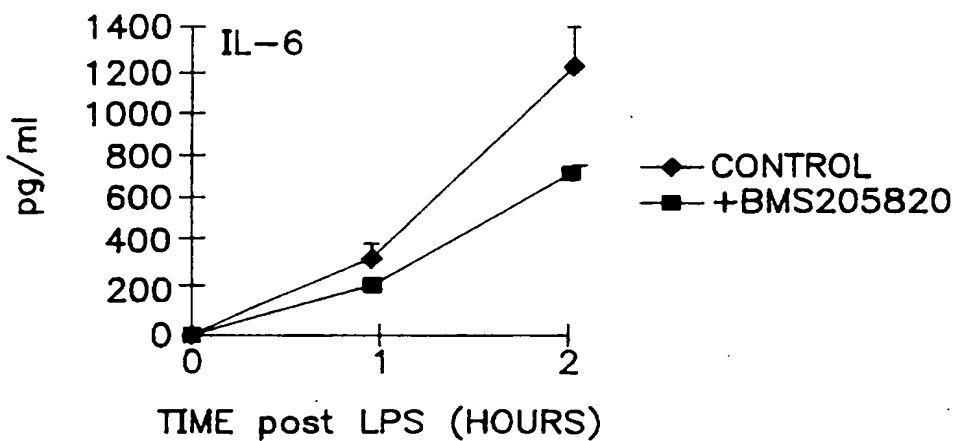
FIG. 9

FIG. 10

FIG. IIA

FIG. IIB

Anti–SRBC IgG Titer

7000 6000 5000 4000 3000 2000 1000 0

CONTROL

Treatment

1 mg/kg × 5d (oral)

FIG. I2

TNF

3500
3000
2500
2000
1500
1000
500
0

pg/ml

◆ CONTROL
■ +BMS205820

0   1   2

TIME post LPS (HOURS)

FIG. 13A

1400
1200
1000
800
600
400
200
0

IL−6

pg/ml

◆ CONTROL
■ +BMS205820

0   1   2

TIME post LPS (HOURS)

FIG. 13B

1600
1400
1200
1000
800
600
400
200
0

IL−10

pg/ml

◆ CONTROL
■ +BMS205820

0   1   2

TIME post LPS (HOURS)

FIG. 13C

SUBSTITUTE SHEET (RULE 26)

FIG. 14

FIG. I5A

FIG. I5B
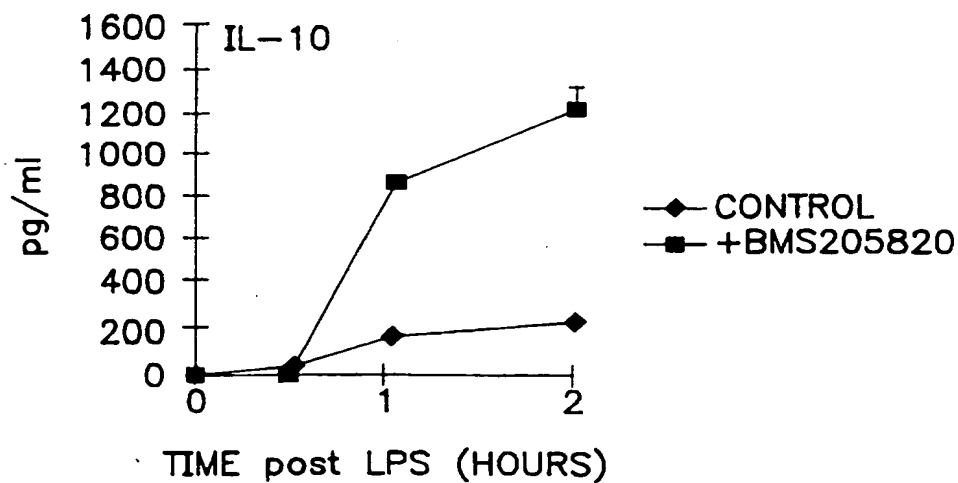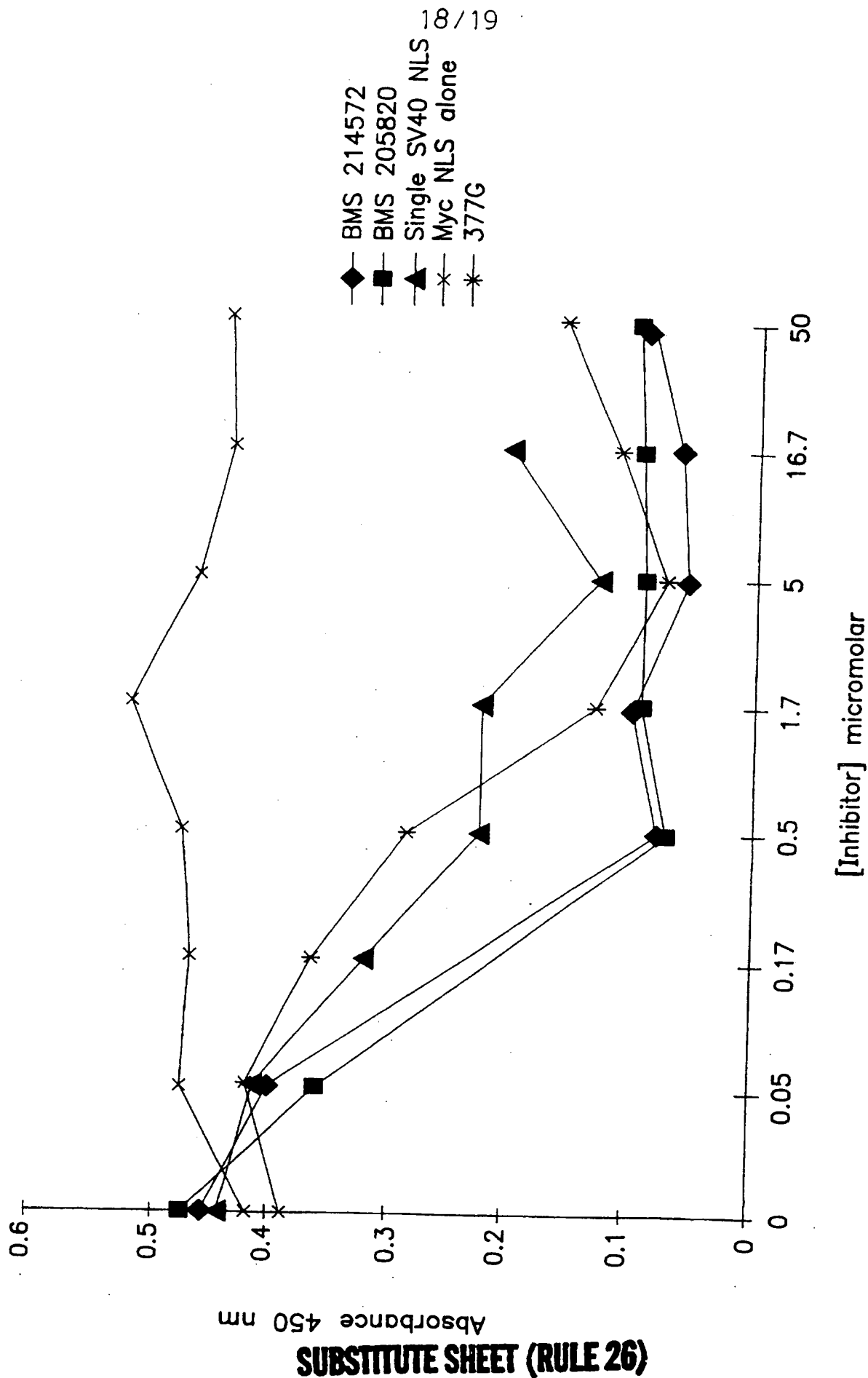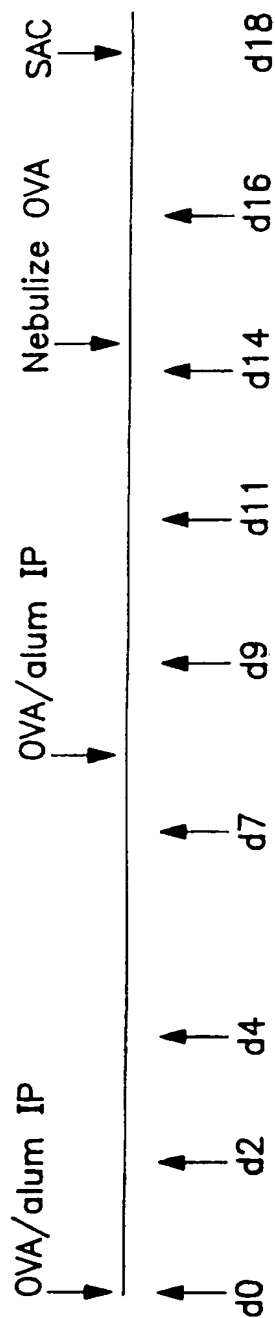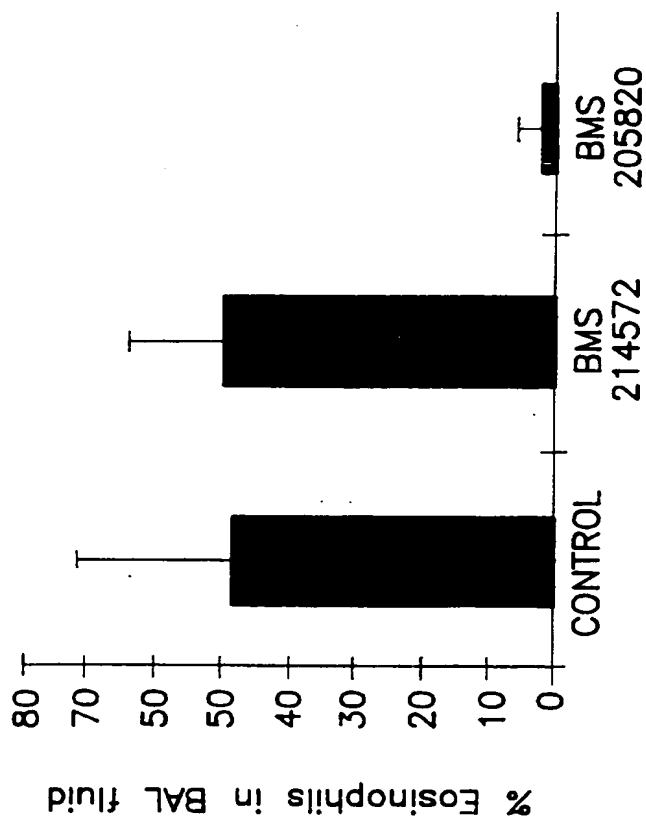
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/02, 38/04, 38/16; C07K 7/06, 7/08

US CL :514/2; 530/300, 326, 327

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2; 530/300, 326, 327

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

SEQ Search, APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94/04696 A1 (MILES, INC.) 03 March 1994 (03-03-94), see especially the abstract and pages 7-8.	1-15
X, P	BERLOSE et al. Conformational and associative behaviours of the third helix of antennapedia homeodomain in membrane-mimetic environments. European Journal of Biochemistry. December 1996, Vol. 242, pages 372-386, especially the introduction and the discussion.	1-15

| X | Further documents are listed in the continuation of Box C. | ☐ See patent family annex. |

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 DECEMBER 1997	1 4 JAN 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	WILLIAM SANDALS
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KENTRUP et al. Dyrk, a dual specificity protein kinase with unique structural features whose activity is dependent on tyrosine residues between subdomains VII and VIII. The Journal of Biological Chemistry. 16 February 1996, Vol. 271, No. 7, pages 3488-3495, see especially the abstract.	1-15
Y	MUKAIGAWA et al. Two signals mediate nuclear localization of influenza virus (A/WSN/33) polymerase basic protein 2. Journal of Virology. January 1991, Vol. 65, No. 1, pages 254-253, see entire article.	1-15
Y	HINKES et al. Organization and promoter activity of the mouse syndecan-1 gene. Journal of Biological Chemistry. 25 May 1993, Vol. 268, No. 15, pages 11440-11448, see especially the abstract and introduction.	1-15
Y	IMAMURA et al. Identification of a heparin-binding growth factor-1 nuclear translocation sequence by deletion mutation analysis. Journal of Biological Chemistry. 15 March 1992, Vol. 267, No. 8, pages 5676-5679, see especially the abstract.	1-15
Y	PEREZ et al. Antennapedia homeobox as a signal for the cellular internalization and nuclear addressing of a small exogenous peptide. Journal of Cell Science. 17 August 1992, Vol. 102, pages 717-722, see the entire article.	1-15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/16217

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 16-24
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)＊